Molecular detection of *Mycobacterium tuberculosis* in stool of children with suspected intrathoracic tuberculosis

by

Corné Bosch

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UNIVERSITY

Supervisor: Dr Kim Hoek

Co-supervisor: Dr Elisabetta Walters

Co-supervisor: Prof Robin Warren

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Declaration

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Abstract

The bacteriological confirmation of tuberculosis in children is challenging. The current diagnostic gold standard, liquid culture of respiratory specimens, has low sensitivity in paucibacillary paediatric tuberculosis, and sputum collection in young children is relatively invasive and resource-intensive. Stool is easy to collect and may contain mycobacterial deoxyribonucleic acid (DNA) from swallowed sputum. However, the performance of polymerase chain reaction (PCR) assays, including Xpert MTB/RIF and HAIN FluoroType may be affected by PCR inhibition from stool enzymes and by instrument failure due to particulate matter blocking filters.

In this study, we aimed to evaluate the diagnostic performance of stool specimens using a variety of stool pre-processing steps, including decontamination and lyophilisation; as well as various DNA extraction and molecular detection protocols.

This study formed part of a larger prospective study involving children with suspected intrathoracic tuberculosis where up to 6 respiratory specimens were collected. Stool specimens were collected at enrolment where one portion was tested by a direct Xpert MTB/RIF protocol; the second portion was frozen for lyophilisation and/or DNA extraction protocols followed by PCR-based molecular detection.

DNA was extracted from stools using either a manual commercial stool or soil kit. Extracted DNA was tested for the presence of mycobacterial DNA using the Xpert MTB/RIF cartridge according to standard manufacturer's protocol and/or a modified "Tube Fill" protocol; and/or the HAIN FluoroType® MTB assay. The results were compared to a composite reference standard and a secondary reference standard (first respiratory culture), which was a better reflection of true performance in our setting.

Our results indicate that the standard and Tube Fill Xpert MTB/RIF protocols, as well as the FluoroType MTB detection platforms are able to detect mycobacterial DNA from stool specimens. The Xpert MTB/RIF performed directly on decontaminated stool specimens was found to have the best diagnostic accuracy with sensitivities of 45.8% - 47.1% and specificities of 97.8% - 98.2%. This method was also found to have the lowest indeterminate rate of 3.4% - 10.3%. The other protocols investigated displayed unacceptable sensitivity and specificity combinations with high rates of indeterminate results. The high indeterminate rates were concerning and further optimisation and method simplification are required to propose stool as a non-invasive specimen type for the rapid confirmation of TB in children.

Opsomming

Die bakteriologiese bevestiging van tuberkulose in kinders is uitdagend. Kultuur van die organisme, *Mycobacterium tuberculosis,* is die huidige goue-standaardtoets vir diagnose van tuberkulose. Ongelukkig is die kultuur van respiratoriese monsters in pediatriese pasiënte vermoeilik, aangesien die laer organismelading van pediatriese tuberkulose en die probleme met sputum versameling in jong kinders. Ontlasting (stoelgang) is maklik om te versamel en kan moontlik mikobakteriële deoksiribonukleïensuur (DNS) bevat vanaf die ingeslukte sputum. Die sukses van die polymerase ketting reaksie (PKR)-gebaseerde toetse, insluitend die Xpert MTB/RIF en die HAIN FluoroType kan egter nadelig beinvloed word deur PKR-inhibeerders teenwoordig in stoelgang (bv. ensieme), asook apparaat wanfunksionering as gevolg van stoelgang restes wat die filtreerders blok.

Die doel van hierdie studie was om die diagnostiese benut van stoelgang as monstertipe vir die diagnose van tuberkulose te bepaal. Verskeie stoelgangs voorbereiding stappe, insluitend dekontaminasie en vriesdroging; asook verskeie DNS ekstraksie en molekulêre opsporingsmetodes is ondersoek.

Die studie was deel van ‘n omvattende studie wat tot en met 6 respiratoriese monsters van kinders met vermoede pulmonale tuberkulose geneem het. Vir die doel van ons studie, is stoelgang monsters aan die begin van die studie versamel, en een porsie is deurmiddel van ‘n Xpert MTB/RIF getoets en ’n tweede porsie is gevries vir latere vriesdroging gevolg deur DNS ekstraksie en PKR-gebaseerde molekulêre opsporing.

DNS is geëkstraheer vanaf stoelgang monsters deur die gebruik van ’n geoutomatiseerde kommersiële stoelgang of grond ekstraksie kit. Geëkstraheerde DNS is getoets vir die teenwoordigheid van mikobakteriële DNS deur gebruik te maak die Xpert MTB/RIF toets volgens die standaard protokol as ook ‘n aangepasde “Tube Fill” tegniek. Die Hain Fluorotype® MTB metode is ook ondersoek. Uitslae van die verskeie metodes is vergelyk met ‘n saamgestelde verwysingstandaard asook ‘n sekondêre verwysingstandaard (die eerste respiratoriese monster), wat ‘n beter besinning is van wat in praktyk in ons omgewing gebeur.

Die studieresultate toon aan dat die standaard en “Tube Fill” Xpert MTB/RIF protokole, asook die HAIN FluoroType MTB deteksie platvorm wel mikobakteriële DNS vanaf stoelgang monsters kan opspoor. Die Xpert MTB/RIF gedoen op gedekontamineerde stoelgang het die beste sensitiwiteit (45.8% - 47.1%) en spesifisiteit (97.8% - 98.2%) opgelewer met ‘n onbepaaldheids persentasie van 3.4% - 10.3%. Die sensitiwiteit en spesifisiteit van elk van die ander protokole was nie belowend nie, en die

metodes het ook hoë onpebaaldheids syfers getoon. Die hoeveelheid monsters wat nie ‘n resultaat kon oplewer nie was kommerwekkend en verg verdere ondersoek om die tegnieke te verbeter en te vereenvoudig. Verdere studies is dus nodig voor stoelgang as ‘n nie-indringende monstertipe vir die spoedige bevestiging van tuberkulose in kinders voorgestel kan word.

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List of abbreviations

AFB	acid-fast bacilli
AM	amplification mix
BAL	bronchoalveolar lavage
BAP	blood agar plate
BCG	Bacille Calmette-Guerin
BSL-2	biosafety level 2
CDC	Centres for Disease Control and Prevention
CFU	colony forming unit
CXR	chest radiograph
DNA	deoxyribonucleic acid
DOTS	direct observed treatment short-course
DST	drug susceptibility testing
ES	expectorated sputum
FNA	fine needle aspirate
GA	gastric aspirate
GP	Green Point
GXT	GenoXtract
HIV	human immunodeficiency virus
IGRA	interferon gamma release assay
INH	isoniazid
IS	induced sputum

IUATLD	International Union Against Tuberculosis and Lung Disease
LAM	lipoarabinomannan
LED	light emitting diode
LIS	laboratory information system
LJ	Löwenstein-Jensen
MDR	multi-drug resistant
MGIT	Mycobacteria Growth Indicator Tube
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
NALC	N-acetyl-L-cysteine
NaOH	sodium hydroxide
NHLS	National Health Laboratory Service
NPA	nasopharyngeal aspirate
NTM	non-tuberculous mycobacteria
OADC	oleic acid albumin dextrose catalase complex
PANTA	Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin
PBS	phosphate buffered saline
PCC	probe check control
PCR	polymerase chain reaction
PTB	pulmonary tuberculosis
PPD	purified protein derivative
RIF	rifampicin
SANAS	South African National Accreditation System

SPC	sample processing control
TB	tuberculosis
TTD	time to detection
TST	tuberculin skin test
WHO	World Health Organisation
XDR	extensively drug resistant
Xpert	Xpert MTB/RIF Assay
ZN	Ziehl-Neelsen

CHAPTER ONE: Introduction

1.1 Background

Despite being one of the oldest infectious diseases affecting human kind, tuberculosis (TB) continues to infect millions of individuals every year, exposing those infected to the risk of progression to active TB disease. Although TB is preventable and treatable, complex socio-economic and health-related factors interact to fuel the ongoing global TB epidemic. The World Health Organisation (WHO) estimates that in 2015 there were 10.4 million new TB cases diagnosed worldwide of which 1 million (10%) were children. The six countries that accounted for the majority (60%) of new cases were India, Indonesia, China, Nigeria, Pakistan and South Africa (1).

Delayed diagnosis of TB and poor implementation and management of the DOTS (Direct Observed Treatment Short-course) programme (2) contribute to the high morbidity and mortality of TB, and promote the emergence and spread of drug resistance in communities. Multi-drug resistant TB (MDR-TB) (3) is characterised by resistance to rifampicin (RIF) and isoniazid (INH), 2 of the critical first line agents. Further resistance can lead to extensively drug resistant (XDR) TB (4), which is resistant to RIF, INH, a fluoroquinolone and an aminoglycoside; and eventually resistance to all known TB drugs (Totally drug-resistant TB (TDR-TB)) (5).

In 2015, South Africa had one of the highest TB incidence rates on the continent and globally, with an estimated total incidence of 834 and 37 per 100 000 population for TB and MDR-TB respectively (1). A further concern in Sub-Saharan Africa, South Africa in particular, is the high rate of human immunodeficiency virus (HIV) and TB co-infection. It is estimated that 6.19 million individuals in South Africa are infected with HIV, of whom 0.26 million are co-infected with TB (1). HIV infection results in dysfunction of cellular immunity, which is the human body's primary mechanism for containment of TB infection. HIV co-infection also complicates TB diagnosis, as described in section 1.2. There were an estimated 1.4 million deaths from TB in 2015 and an additional 0.4 million from TB among people living with HIV. In children, there were an estimated 0.17 million deaths from TB and 0.04 million deaths among HIV-infected children. Despite the decline in TB deaths from 2000 – 2015 by 22%, TB remains one of the top 10 causes of death worldwide (1).

High TB-burden regions are often poorly resourced: limited access to healthcare and health education may lead to a low awareness of TB symptoms (6). Stigma towards infected individuals is also a widespread cause of delayed diagnosis and treatment, with patients avoiding reporting of symptoms (7). A definitive diagnosis of TB depends on laboratory confirmation, but healthcare services are often poorly managed (8) and current diagnostic methods are suboptimal due to poor sensitivity and long turnaround time to results, particularly in high-risk populations such as HIV-infected individuals and children. The low bacillary load in HIV-infected individuals and in children translates into low sensitivity of smear microscopy, culture and molecular detection tests. In addition, diagnosis is further complicated by the difficulty in collecting high quality specimens for testing in sputum-scarce adults and in young children. Diagnostic delay leads to increased morbidity and mortality from TB, along with increased risk of spreading the disease in the community.

1.2 Tuberculosis disease

TB disease is caused by a group of closely related mycobacteria collectively classified as *Mycobacterium tuberculosis* complex (MTBC). The complex consists of *M. bovis*, *M. africanum*, *M. microti*, *M. canetti* and, the most prevalent, *M. tuberculosis* (MTB). The causative organisms, MTB, are rod-shaped aerobic bacteria that possess a mycolic acid-rich cell wall structure and are classified as non-spore forming, strictly aerobic acid-fast bacilli (AFB). The exceptional lipid structure of the cell wall provides an extensive protective barrier against antibiotics and the cellular defence mechanisms of the host (9). MTB is an airborne pathogen that can easily spread from person to person by the inhalation of small droplet nuclei (1-5 µm in diameter) containing viable MTB bacilli (10). These infectious droplet nuclei can enter the air when a person with active pulmonary tuberculosis (PTB) coughs, sneezes, or during aerosolisation of respiratory secretions during specimen collection (e.g. during sputum induction). The risk of infection is dependent on a variety of factors such as the mycobacterial load (smear positivity) of the source case, the relative proximity of individuals, indoor ventilation and the duration of exposure (11). The nuclei droplets are small enough to enter into the alveoli where the mycobacteria can either be cleared by the immune system, remain dormant (latent TB infection) or systematically cause active pulmonary disease within the alveolar macrophage that ingests it (12). Once MTB is ingested by a macrophage, the bacilli release proteins that interact and prevent fusion with the lysosome and MTB can thus proliferate creating a localised (primary) infection. A few weeks after a primary infection has occurred, other immune cells are attracted to the infected

area, and, through cell-mediated immunity, create a granuloma. The tissue located in the centre of the granuloma dies during a process called caseous necrosis and forms scar tissue known as a Ranke-complex, which may be visible on a chest radiograph (CXR). The immune system attempts to isolate the dead tissue but the MTB bacilli within can remain viable. When the immune system becomes compromised by old age, HIV-infection, malnutrition or the use of immunosuppressant drugs, the bacilli can re-activate and spread through the lung causing the host to release more inflammatory mediators, thereby resulting in an increase in caseous necrosis. This widespread necrosis leads to cavity formation within the lung. MTB can also proliferate and disseminate throughout the lung to other organs via the blood stream or lymphatic system, resulting in extrapulmonary or miliary TB disease (13).

Several factors contribute to the complexity of the clinical manifestations of TB, including age, immune status, nutrition, comorbidity, genetic factors, virulence of the organism and the site of disease. The symptoms of adult-type TB are usually systematic in commencement and the duration can vary from weeks to months. The most common clinical symptoms include prolonged cough, fever, night sweats, weight loss and haemoptysis (14). TB presentation is frequently atypical in HIV-infected individuals. HIV infection is associated with increased risk of active TB following new or latent TB infection and of TB recurrence, compared to HIV-uninfected individuals (15). HIV-associated TB is less likely to present the characteristic clinical and radiological findings usually associated with active TB and is often sputum smear microscopy negative resulting in delayed diagnosis and treatment (16).

The clinical presentation of TB in children is frequently non-specific, although older children may show signs and symptoms similar to adult-type TB (17). However, the greatest risk for TB-related morbidity and mortality remains in very young children as they are at higher risk of developing active TB after primary infection. In this population group, TB symptoms are non-specific and overlap with many other conditions, especially in HIV-infected children. The most common general symptoms are fever, lethargy and weight loss or poor growth, while the most common organ-specific symptom is persistent cough lasting more than 2 weeks (18). However, in children younger than 3 years, the presentation may be more acute (19).

1.3 Diagnosis in children in South Africa

A definite diagnosis of TB is challenging in children due to the paucibacillary nature of the disease. Sputum smear microscopy provides confirmation of AFB in only 10 - 15% of children with TB disease. The gold standard, culture-based method, is more sensitive than smear microscopy (30 - 40%), but most children treated for TB are culture negative (i.e. clinically diagnosed) (20,21). In children, PTB can be confidently diagnosed in a proportion who present with typical symptoms, suggestive CXR and evidence of TB infection (see immunological tests in section 1.3.3). However, in most children, not all factors may be present. In young children who are unable to expectorate spontaneously, the collection of adequate respiratory specimens is challenging and requires relatively invasive and resource-intensive procedures.

1.3.1 Clinical investigations

In non-endemic regions, a positive tuberculin skin test (TST), a suggestive CXR and exposure to a confirmed known source is often sufficient to diagnose TB disease in children. In endemic regions, however, community exposure to TB is much higher and these methods show limited value in diagnosing and differentiating between active and latent TB disease. Symptom-based approaches to diagnose childhood TB have poor diagnostic accuracy, as the disease spectrum is often broad and the non-specific symptoms displayed by children with TB could be due to a variety of other unrelated illnesses. Well-defined symptoms such as persistent non-remitting cough or wheezing, failure to thrive and fatigue or reduced playfulness were found to have a sensitivity of 82.3% for diagnosing TB in children >3 years of age, but performed poorly in young and HIV-infected children (18). Due to the limitations of the different diagnostic modalities and low sensitivity of bacteriological tests in children, various scoring methods have been developed to diagnose active TB. However, these scoring systems have not been validated and vary widely in performance depending on setting and patient population (22).

1.3.2 Radiological methods

Chest radiography is widely used to aid in the diagnosis of childhood TB, where radiology services are available and affordable. Chest radiographs are useful to diagnose typical manifestations of TB such as miliary TB and effusion. However, radiography is highly operator dependant and the interpretation has high inter-reader variability (23), especially to detect hilar nodes, which is the most common manifestation of TB in children. In addition, non-TB conditions can present very similar radiographic findings. This is especially the case in children with HIV-associated lung disease. The interpretation of results remains subjective and usually requires one or more experienced clinicians to accurately and correctly interpret results (24). In children, computed tomography is useful for detecting intrathoracic lymph nodes, airway compression and differentiating TB from other conditions (25). However, this diagnostic modality is unaffordable for most low-resource settings. Radiographic tests cannot be used as an independent diagnostic tool due variable disease presentation, interpretation of results and the similarity of patterns associated with other non-TB diseases (20).

1.3.3 Immunological methods

Immune based assays can be used to detect antibodies, antigens and immune complexes. Serological assays for TB have demonstrated very poor diagnostic utility and the WHO has issued strong recommendations against the commercial use of serodiagnostic tests for diagnosis of active TB (26). Serological assays are mainly used to demonstrate exposure to TB although in high endemic areas the need remains for the clear differentiation between active and latent TB (20). Tests of infection in current use are the Mantoux TST and Interferon Gamma Release Assays (IGRAs).

TST uses a purified protein derivative (PPD) derived from tuberculin, which is injected under the skin (intradermally). If the PPD is recognised by the immune system, it induces a delayed T-cell mediated reaction in most infected individuals. The TST is evaluated 48-72 hours after administration. Recently infected individuals may be non-reactive for 2-8 weeks post infection. The TST cannot differentiate between TB infection and active TB disease. In addition, several factors can result in false positive results, such as infection with some species of non-tuberculous mycobacteria (NTM) and Bacille Calmette-Guerin (BCG) vaccination. False negative results can occur in individuals with a recent

primary infection, HIV-infected individuals, overwhelming TB infections, very young children and the incorrect administration and interpretation of the TST (27).

Interferon Gamma Release Assays (IGRAs) determine the presence of TB infection by measuring the immune response to TB proteins ESAT-6 and CFP-10 (with or without TB 7.7) in whole blood. IGRAs do not differentiate between latent and active disease and additional tests are required to confirm TB disease. The tests are expensive compared to the TST although they do seem to perform better in HIV associated TB (28).

1.3.4 Bacteriological methods

The most common procedure to obtain swallowed sputum from infants and young children suspected of having TB is the aspiration of gastric contents, as young children are unable to spontaneously expectorate sputum (29). In our setting, it is recommended that at least two gastric aspirate (GA) specimens are collected on consecutive days (30) from patients who cannot expectorate, although guidelines from the WHO and Centres for Disease Control and Prevention (CDC) recommend 3 consecutive GA specimens. GA specimens are usually collected early in the morning, before gastric emptying and following an overnight fast (31). Sputum induction, which does not require hospitalisation, and less invasive nasopharyngeal aspiration (NPA) have also been used successfully for TB diagnosis in community-based studies. Bacteriological detection from induced sputum (IS) may be comparable to that from GA specimens (32–34). NPA specimens are easier to collect than IS, but diagnostic performance appears inferior (35). The collection of multiple specimens of different types has been shown to result in higher bacteriological detection than the collection of numerous specimens of the same type (35). There are numerous bacteriological methods for the detection of MTB, all with varying sensitivity. We discuss the methods routinely used in the study setting.

a) Microscopy

Sputum smear microscopy is considered an inexpensive, rapid and relatively straightforward diagnostic test, especially in high TB-burden regions. However, sensitivity in children remains low with fewer than 20% of TB cases being smear-positive (36,37). Since its introduction in 1937 (38),

fluorescence microscopy has been widely used as a rapid detection method of acid fast bacilli. Fluorescence microscopy uses an acid-fast dye, usually Auramine O or Auramine-rhodamine, in combination with an intense light source such as a halogen, mercury vapour or light emitting diode (LED) lamp. The conventional Ziehl-Neelsen (ZN) staining is less sensitive than fluorescent staining for direct microscopy and is now generally used to detect the presence of AFB in positive cultures. Sputum concentration prior to microscopy also shows increased sensitivity (average 18%) over direct microscopy from unconcentrated sputum (39).

AFB microscopy detects both viable and non-viable bacilli that have been stained and counterstained on a glass slide and involves the physical counting of bacilli to report results based on a WHO/International Union Against Tuberculosis and Lung Disease (IUATLD) grading system (40). Other Gram-negative bacteria, including *Nocardia* and *Corynebacteria* species may also stain positive, thereby affecting the specificity of the test. AFB microscopy can therefore not be used as a stand-alone test to confirm a diagnosis of TB disease. Furthermore, the analytical sensitivity of microscopy is only ± 10^4 bacilli per millilitre and therefore many true positives are missed by microscopy alone.

ZN stains are prepared from MGIT positive cultures. A smear of the culture is prepared on a glass microscope slide and is stained using a combination of carbol fuchsin (AFB stain), heat, acid alcohol (decolouriser) and methylene blue (counterstain) as per the procedure in Section 2.7.5. AFB stain red and the majority of MTBC strains form cord-like (serpentine) structures when viewed under a light microscope. Where no AFB are seen and/or where other micro-organisms are visible, samples are re-decontaminated as per Section 2.7.1. Suspected false positives are eliminated according the NHLS TB laboratory algorithm (Appendix G).

b) Culture

Despite recent advances in molecular based detection platforms, the gold standard for MTB detection remains culture. The main benefit of a culture based system is the ability to perform multiple tests on a single isolate if required.

TB culture includes the following aspects:

1) inhibition of all bacteria other than mycobacteria (decontamination),

2) separating/releasing mycobacteria from the specimen e.g. thick mucus from respiratory specimens or solid organic cellular debris found in stool specimens (liquefaction or digestion); and;

3) the growth of the mycobacteria for further diagnostic identification.

The sodium hydroxide/N-acetyl-L-cysteine-sodium citrate (NaOH-NALC-Na citrate) method is recommended for decontamination of respiratory specimens. NALC is a mucolytic agent, while NaOH is a decontaminating agent at low (final) concentrations (1 - 1.5%) and Na-citrate acts as a stabilisation agent on the NALC. NaOH, however, remains toxic to contaminating microorganisms and mycobacteria at high concentrations or prolonged exposure and is neutralised by the addition of phosphate buffered saline (PBS).

The growth of mycobacteria is achieved by using the Bactec MGIT 960 system (Becton Dickinson diagnostic systems, New Jersey, USA) where the sediment obtained after decontamination and digestion is inoculated into a Mycobacteria Growth Indicator Tube (MGIT). The Middlebrook 7H9 broth inside the MGIT is nutrient rich and in combination with an added growth supplement, oleic acid albumin dextrose catalase complex (OADC), and antibiotic mixture, Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin (PANTA), provides a suitable environment for the culture of mycobacteria. Detection of mycobacterial growth is based on a fluorescent indicator compound embedded within the MGIT's silicon rubber base which is oxygen sensitive. As bacteria metabolise the available (free) oxygen in the MGIT, the carbon dioxide levels increase and the fluorescence levels rise as the fluorescent indicator is no longer inhibited by the oxygen, resulting in a positive signal. The turbidity of the growth can be visually assessed: mycobacteria will be granular or flaky in appearance and not very turbid, whereas bacterial growth is highly turbid. The time to detection (TTD) of adequate fluorescence (average of 8 to 14 days in this setting), usually between 10^5 or 10^6 colony forming unit (CFU) per millilitre (41), is recorded by the on-board software and provides an indication of the initial bacterial concentration of the original specimen. The average TTD is dependent on the sample type, contamination rates and is often longer for paediatric TB specimens due to the low bacillary load. In most settings, once a positive signal (when the growth unit reaches 400) is observed (42), confirmation of positivity is done by ZN microscopy (Section 1.3.4) and inoculation on a blood agar plate (BAP) to detect contamination. A rapid antigen identification test, such as the MPT64 lateral flow immunochromatographic assay (43) is used to confirm the presence of MTB complex in a culture (38) when no further drug susceptibility testing (DST) is requested or to confirm an NTM or BCG infection (Appendix G).

It is recommended to use more than one type of selective media to maximise the recovery of MTB from clinical specimens. This is most commonly additional solid culture medium, usually an egg-based medium for example an LJ slope, Ogawa or Middlebrook (7H10, 7H11) media. However, in our setting, solid culture media are not used to culture TB specimens.

There is usually a diagnostic delay associated with all culture-based methods for the detection of drug-resistant paucibacillary TB: the delays could be patient-related (late presentation of symptoms) or doctor-related (failure to consider a diagnosis of tuberculosis). Studies on the exact impact of these delays on paediatric TB diagnosis are limited although some evidence suggests that they adversely impact the time to appropriate treatment (44) and could affect clinical outcomes (45).

c) Phenotypic drug susceptibility testing (DST)

Phenotypic DST typically requires a positive culture (indirect method), where growth (solid) or a cellular suspension of MTB growth (liquid) is inoculated on solid media or in liquid (broth) media containing a pre-determined concentration of a targeted antimicrobial agent. The traditional DST method, the agar proportion method, uses a homogenous suspension of confirmed TB positive cells that are inoculated onto calculated antimicrobial agent-containing and antimicrobial agent-free (control) solid media agar plates (Middlebrook). The ratio of CFUs (antimicrobial agent-containing vs. antimicrobial agent-free solid media) determines the susceptibility or the resistance of the isolate to a specific antimicrobial agent.

The more commonly used method is the broth based or liquid culture proportion method using either the BACTEC460 (Becton Dickinson diagnostic systems, New Jersey, USA) or the MGIT960 system. Similarly, a confirmed TB positive cellular suspension is inoculated into a calculated antimicrobial agent-containing and antimicrobial agent-free (control) broth tube. The cellular suspension must be tested within 1-5 days of instrument positivity for MGIT cultures; cultures that have been positive for more than 5 days must first be subcultured into a fresh MGIT prior to phenotypic DST. The positive cellular suspension (0.5 ml) is added to 5 MGIT tubes containing 0.8 ml MGIT SIRE supplement as well as 0.1 ml of the different drugs tested for; the growth control (GC) tube will not have any drug added. The 5 tubes are then incubated in the MGIT 960 instrument that monitors the susceptibility test set. The instrument interprets the results when the growth unit in the GC tube reaches 400 (within 4-13 days). If the GC tube becomes positive within 4 days or remains negative after 13 days of incubation,

the test is repeated by either increasing or decreasing the dilution of the original culture, respectively (41). Following incubation (4-21 days), a comparison of the fluorescence emitted by both tubes concludes whether a specimen is susceptible (the growth unit of the drug-containing tube is less than 100) or resistant (the growth unit of the drug-containing tube is more than or equal to 100) to a specific antimicrobial agent. Each antimicrobial agent has a precise critical concentration and increased microbial growth, solid or liquid based, at this concentration, when compared to the same isolate diluted to a defined concentration in the absence of the antimicrobial agent, will be considered resistant (41).

1.3.5 Genotypic methods

In the South African public healthcare system, two different PCR-based methods are available for the diagnosis of TB and associated drug resistance: the GeneXpert MTB/RIF assay (Xpert; Cepheid, Sunnyvale USA) and the Genotype MTBDR*plus* and MTBDR*sl* assays (Hain Lifescience GmbH, Nehren, Germany). The Xpert MTB/RIF assay is a WHO-recommended rapid (within 2 hours) diagnostic test for the detection of PTB and RIF resistance from primary clinical specimens from adults (since 2010) and children (since 2013). The Xpert assay is an automated diagnostic test and a semi-quantitative, nested real-time PCR used for the detection of MTB DNA. The assay is recommended for use in combination with other routine clinical and laboratory tests on patients with suspected TB with no, or less than three days of antituberculosis treatment (46). The Xpert assay targets the 81-base pair core region of the *rpo*B gene, which contains mutations associated with RIF resistance (detectable by 5 wild-type and 3 mutation probes). The assay can be used on raw sputum specimens or concentrated sputum sediments from IS or expectorated (ES) sputa. Specimens are tested using disposable single-use cartridges and results are interpreted by the GeneXpert Dx software after testing on the GeneXpert instrument. The automated process also includes internal controls for sample processing (SPC) and a probe check (PCC). The SPC contains non-infectious spores and regulates the appropriate processing of target bacteria and monitors the presence of PCR inhibitors whilst the PCC ensures that there is sufficient reagent rehydration, PCR tubes are correctly filled, probes are checked and monitors the stability of the dye. The SPC should always be positive in the case of a negative Xpert MTB/RIF result; however, SPC may be negative in the event of a positive result due to competitive inhibition. If the internal control(s) fail, the test should be repeated if there is sufficient specimen volume remaining (47).

In children with pulmonary TB, the Xpert MTB/RIF assay has 36-44% increased sensitivity compared to smear microscopy; and sensitivity compared to culture is approximately 62% for expectorated sputum (ES), and 66% for both IS and GA (48). The Xpert MTB/RIF assay can also detect more than 99.5% RIF resistance compared to phenotypic DST (49). The WHO also recommends the use of the Xpert MTB/RIF assay on some extrapulmonary specimens including cerebrospinal fluid (CSF), fine needle aspirates (FNA), pleural fluid and tissue specimens (47). The assay can be used directly on CSF, FNA and pleural fluid specimens or homogenised (PBS buffer) tissue biopsy specimens ground by a mortar or pestle, avoiding clumps of tissue being transferred to the cartridge. For extrapulmonary TB, especially in young children, culture is recommended for Xpert MTB/RIF negative specimens. It is recommended that low-volume CSF specimens from children are preferentially tested by Xpert rather than culture (47). For extrapulmonary specimens obtained from adults and children, the Xpert MTB/RIF assay demonstrated sensitivities of 84.9% for lymph node tissue/aspirates, 83.8% for gastric fluids, 81.2% for tissue specimens, 79.5% for CSF and 43.7% for pleural fluid, when compared to liquid culture (47).

The second group of genotypic assays routinely used in the South African context are the Genotype MTBDR*plus* (version 2) and MTBDR*sl* assays (Hain Lifescience GmbH, Nehren, Germany). Both can be done directly from clinical specimens, usually if smear-positive, or on positive culture material (as for our setting) to determine genotypic RIF and INH susceptibilities or to confirm RIF resistance detected by the Xpert assay. The MTBDR*plus* assay simultaneously detects mutations associated with RIF resistance of the *rpo*B gene and INH resistance associated mutations of the *kat*G (high level INH resistance) and *inh*A (low level INH resistance) genes. Mutations causing resistance can be detected in more than 98% of RIF resistant strains and 90% of INH resistant strains (50).

The MTBDR*sl* assay is used for the detection of second-line antimicrobial susceptibility (fluoroquinolones and aminoglycosides) and is a useful tool to rapidly detect XDR-TB when used in combination with the MTBDR*plus* assay. The MTBDR*sl* assay (version 2.0) detects mutations in the *gyr*A and *rrs* genes determining susceptibility to the fluoroquinolones and the second-line injectable drugs kanamycin/amikacin respectively. Fluoroquinolone resistance caused by mutations in the *gyr*A gene accounts for 75 - 95% and aminoglycoside resistance caused by mutations in the *rrs* gene accounts for ~76% of resistance (51). The Xpert MTB/RIF assay, along with all other molecular based tests cannot be used to monitor treatment outcomes due to the detection of MTB DNA which could originate from either live or dead bacilli. There are limited data on the use of the MTBDR*plus* and MTBDR*sl* assays on extrapulmonary specimens as they were designed for direct sputum samples.

Other specimen types (Bronchoalveolar lavage - BAL, CSF or other body fluids) have not been comprehensively evaluated (52,53).

1.4 Alternative specimen types

The difficulty in obtaining representative specimens from young children and those unable to expectorate remains challenging and extrapulmonary sites may not always be accessible. Due to the difficulties in obtaining serial GA specimens, alternative specimen types for the detection of PTB include BAL, gastric fluid absorbed by a string, NPA and stool specimens, bone marrow and urine have also undergone evaluation for the diagnosis of TB when extrapulmonary TB is suspected (30).

BAL specimens are obtained using flexible bronchoscopy, during which lavage fluid is flushed into the suspected diseased portion of the lung and is aspirated by suctioning. Bronchoscopy is invasive, expensive and requires specialised personnel and equipment. BAL specimens have shown to have a lower yield compared to GA and IS for TB culture (54,55) in children with uncomplicated PTB. More recently, BAL specimens showed an incremental diagnostic yield (compared to culture of routine respiratory samples) in a small subset of children with complicated intrathoracic TB using the Xpert MTB/RIF assay (56,57).

NPA specimens are obtained by inserting a tube through the nostril into the nasopharynx after instillation of saline solution into the nose, followed by aspiration or expectoration of secretions from the lower respiratory tract. This method is considered minimally invasive and does not require a lengthy fasting period or hospitalisation. Early studies showed that the diagnostic yield from NPA was comparable to that of GA or IS specimens (54). Further studies showed the bacteriological yield to be lower compared to GA specimens (58,59). Molecular detection in children with suspected PTB showed that the use of two NPAs for Xpert testing could be useful in settings where culture and IS are unavailable (35).

The string test collection method uses an encapsulated absorbent nylon string that must be swallowed by an individual allowing it to enter the stomach. The latter half of the string remains fastened to the cheek and the string is recovered by gentle traction after 2-4 hours and placed in a saline buffer (60). The string test demonstrated the ability to successfully retrieve MTB from the stomach of sputum

scarce adults (61). In children the string test detection yields were shown to be comparable to IS although many children (16.1%) were unable to swallow the large capsule (60).

FNA is considered minimally invasive and involves the use of a fine 23-gauge needle to obtain an aspiration from a lymph node for the diagnosis of tuberculous lymphadenitis, the most common form of extrapulmonary TB disease in children (26). Diagnostic yield from suspected TB lymph nodes is high on most modalities, including culture and Xpert (62). However, this procedure requires a lymph node to be visibly enlarged and highly trained clinical staff to complete the procedure (54).

Bone marrow biopsy and aspiration for the diagnosis of extrapulmonary TB are infrequently used and typically reserved for situations where the clinical suspicion of disseminated TB is strong, all other less invasive tests were inconclusive or if a confirmed diagnosis would influence treatment decisions and patient outcomes (63).

All the above-mentioned specimen collection methods are invasive, somewhat traumatic for children and usually require hospitalisation. For children, the ideal specimen would be one that can be collected easily and non-invasively. Urine and stool are examples of such specimens.

Urine can be collected relatively easily from children using urine collection bags applied to the perineum or containers for children who are toilet trained. The detection of lipoarabinomannan (LAM) using immune-capture assays for diagnosing active TB from urine had been considered initially a potentially revolutionary tool for HIV-associated TB and was accelerated to commercial development. Subsequent larger studies failed to demonstrate adequate sensitivity under routine conditions from non-selective participants (64,65). Despite very limited and low-quality data from children, TB LAM lateral flow testing was recently accepted by WHO as a rule-in test for HIV-infected children only, but should be used in combination with other detection methods (66). The TB LAM test may only be used to assist the diagnosis in HIV positive children or adults with sign or symptoms of TB where they have a CD4 count of less than 100 cell/µl or if they are seriously ill with no CD4 count available (67).

1.4.1 Stool specimens

Stool specimens are easy to collect and can contain intact bacilli from swallowed sputum following the passage through the digestive tract (68,69). Stool contains many PCR inhibiting substances and contaminating bacteria and thus requires stringent decontamination procedures, which could in turn

also affect the viability of MTB and possibly degrade MTB DNA. The different decontamination methods used for the culture of stool specimens are outlined in Table 1.1 below.

Most studies use the conventional NALC-NaOH Na-citrate decontamination method as described by Kent and Kubica in 1985 (70) for respiratory specimens or a variation of this protocol. Due to the vast number of microorganisms found in stool specimens, finding the delicate balance between sufficient bacterial decontamination whilst not eliminating too many or all MTB bacilli remains challenging.

Colenbunders *et al* (68) used three decontamination methods as summarised in Table 1.1 on adult stools and concluded that MTB are less likely to be isolated from patients with diarrhoea than without, most likely due to the dilution of organisms created by the increased water content. However, they did not compare the decontamination methods. Kokuto *et al* (71) used the conventional NALC-NaOH Na-citrate method, with a final NaOH concentration of 3%, to process 2 cm^3 of adult stool specimens in MGIT and 2% Ogawa media. For MGIT and Ogawa culture, sensitivities and specificities were 31.9% and 100%; 21.4% and 100%, respectively. Contamination of MGIT culture was 14.0% as opposed to 0% for Ogawa media. They concluded that the culturing of stool specimens for PTB detection was ineffective (71).

Oberhelman *et al* (58) collected daily stool specimens from children over 2 days and showed that GA specimens were superior to stool specimens for MTB recovery in 15 culture confirmed cases. They suggest that the sensitivity of detection in stool specimens can be increased by culturing a larger volume of specimen if the decontamination and concentration process could be improved.

El Khechine *et al* (72) filtered stool specimens using a faecal specimen filtration vial kit which they modified by the addition of a macro porous compress that has specific mesh openings and uniformly oriented fibres which ensured that mycobacterial cells were not trapped within the filter matrix. Specimens were decontaminated with 3 volumes of 1% chlorhexidine digluconate. They reported MTB culture detection in 14.9% of sputum specimens compared to 9.7% in stools. Stool culture demonstrated a sensitivity of 54.2% and specificity of 100% compared to any confirmed culture positive specimen. Similarly Donald *et al* (69) collected two stool specimens from each participant using the method of Allen *et al* (73), and both studies concluded that stools could be used in conjunction with sputum testing or as an alternative diagnostic specimen (Table 1.1) (69,74).

Table 1.1. Overview of studies using different stool processing methods for mycobacterial culture

Decontamination method	Volume and concentration	Sample size (patients)	Culture media	Stool Culture positive	Sensitivity	Specificity	Contamination rate	Reference standard	Stool amount	Population	HIV	Reference
Chlorhexidine digluconate	3 vol of 1% chlorhexidine digluconate	n = 134	LJ	n = 13	54.2%	100%	Not Stated	Any culture positive (stool or sputa)	2 spoonfuls**	Adults and children	Not Stated	(72)
NALC/NaOH NA-Citrate	0.5% NALC/2% NaOH 1.45% Na-citrate	n = 165	LJ and MGIT	n = 3	20.0%	100%	Not specified for stool	Any culture positive (stool, GA, NPA)	0.1 g	Children	Uninfected	(58)
NALC/NaOH NA-Citrate	0.5% NALC/2% NaOH 1.45% Na-citrate	n = 456	LJ and MGIT	n = 4	18.2%	100%	Not specified for stool	Any culture positive (stool, GA, NPA)	0.1 g	Children	Uninfected	(59)
3 methods*	3 methods*	n = 59	LJ and Ogawa	n = 4	7.0%	Not Stated	Not Stated	HIV related enteritis	Not Stated	Not stated	Infected	(68)
		n = 41	LJ and Ogawa	n = 1	2.0%	Not Stated	Not Stated	None-HIV related enteritis	Not Stated	Not Stated	Uninfected	
Combined 3 methods	Not Stated	n = 276	Liquid Kirchner medium	n = 61	22.0%	Not Stated	Not Stated	Diagnosed PTB	0.5 g suspension	Not Stated	Both	(73)
Allen BW	Not Stated	n = 76	Liquid Kirchner medium	n = 3	5.0%	Not Stated	Not Stated	Suspected pulmonary TB suspects	Not Stated	Children	Not Stated	(69)

Decontamination method	Volumes and concentration	Sample size (patients)	Culture media	Culture positive	Sensitivity	Specificity	Contamination rate	Reference standard	Stool amount	Population	HIV	Reference
NaOH	5 ml of 1mol/1NaOH	n = 276	Liquid Kirchner medium	n = 60	98.0%	Not Stated	Not Stated	Any positive stool culture	0.5 g suspension	Not Stated	Both	(73)
Method of Porteals *et al* (75)	0.2% malachite green, cycloheximide (500mg/l) and 1 mol/l NaOH	n = 276	Liquid Kirchner medium	n = 28	46.0%	Not Stated	Not Stated	Any positive culture	0.5 g suspension	Not Stated	Both	
Benzalkonium chloride (BZK) method	0.1% BZK and 0.1% 1-hexadecylpyridinium (HPC)	n = 276	Liquid Kirchner medium	n = 32	52.0%	Not Stated	Not Stated	Any positive culture	0.5 g suspension	Not Stated	Both	
NALC/NaOH NA-Citrate	Equal volume	n = 192	LJ	n = 8	27.0%	Not Stated	Not Stated	AFB positive stool specimens	2-3 g	Children	Status unknown	(76)
NALC/NaOH	Equal volume of 3% NaOH (final)	n = 93	MGIT	n = 15	31.9%	100%	14.0%	Active PTB	2 cm^3	Adults	Uninfected	(71)
			Ogawa	n = 12	21.4%	100%	0%	Active PTB	2 cm^3	Adults	Uninfected	

*Three methods were used: a) method of Petroff b) the method of Beerwerth and Schurmann c) the method of Wilinsky and Rynearson modified by Portaels *et al* (73).
** No volume specified only defined as “spoonful”

Due to the varied and limited success of stool specimens for both solid and liquid culture-based testing, studies have implemented molecular detection techniques in an attempt to avoid the contaminating effect of the many microorganisms present in the stool. Molecular detection studies are summarised in Table 1.2 below.

Cordova *et al* (77) collected pairs of stool specimens, from both HIV seronegative and seropositive adults (>17 years of age), prior to or within 2 weeks of treatment start date. They demonstrated that, heminested IS*6110*-PCR on stool is a useful method for rapid MTB detection with sensitivity and specificity similar to that of conventional sputum culture (Table 1.2) (77).

Wolf *et al* (78) used stool specimens stored for two years from culture confirmed cases in children. They showed that IS*6110*-PCR with Fast-DNA stool sample processing for diagnosing PTB had a sensitivity of 38% and specificity of 100% compared to culture of multiple specimens. They also showed that culture and PCR testing on duplicate specimens increased diagnostic sensitivity (Table 1.2), suggesting that the mycobacterial load of paediatric specimens oscillates around the threshold of detection sensitivity of current testing platforms (78).

Oberhelman *et al* showed that IS*6110*-PCR was more sensitive for detecting MTB in NPA and GA specimens than from stool specimens in children. They also found that several healthy controls were PCR positive suggesting false-positive results despite rigorous measures to prevent cross contamination. These results could also indicate that PCR may detect early, latent or asymptomatic TB disease. (59).

Hilleman *et al* (28) tested decontaminated stool specimens from adults and children using the Xpert MTB/RIF assay, along with MGIT and Löwenstein-Jensen (LJ) cultures. Besides detecting MTB with 100% sensitivity and 91.7% specificity compared to culture, stool Xpert also detected MTB in 21.7% of culture contaminated specimens (Table 1.2) (79).

Table 1.2. Overview of molecular detection in stool specimens

Molecular detection method	Sample size (tested on stool)	Sensitivity	Specificity	Error/Indeterminate Rate	Reference standard	Study populatiion	Stool used	Stool mass	HIV	Extraction method	Reference
Heminested IS 6110 PCR assay	n = 134	20.2%	97.3%	Not Stated	Clinically diagnosed	Adults and children	Filtered	2 spoonfuls**	Not Stated	Nucleospin tissue minikit	(72)
Heminested IS 6110 PCR assay	n = 70	84.0%	100%	None	Sputum culture positive	Adults (>17 years old)	Raw	Manufactures' instruction	HIV uninfected and infected	QIA DNA extraction	(77)
		64.0%	100%	None	Sputum culture positive	Adults (>17 years old)	Decontaminated	0.1 g		chelex and QIA DNA extraction	
Heminested IS 6110 PCR assay	n = 39	38.0% (6/16)	100%	None	Culture positive	Children	Raw and stored	0.2 g	HIV uninfected	Fast DNA extration	(78)
		31.0% (5/16)	100%	None	Culture positive	Children	Decontaminated	0.2 g		Chelex extraction	
Heminested IS 6110 PCR assay	n = 148	20.0% (4/20)	93.0%	None	Any culture positive	Children	Decontaminated stool	0.1 g	HIV uninfected	Not Stated	(59)

Molecular detection method	Sample size (tested on stool)	Sensitivity	Specificity	Error/Indeterminate Rate	Reference standard	Study populatiion	Stool used	Stool mass	HIV	Extraction method	Reference
Xpert MTB/RIF	n = 23	100% (2/2)	91.7%	13.0%	Stool culture confirmed	Adults and children	Decontaminated	Not Stated	Not Stated	Xpert MTB/RIF	(79)
Xpert MTB/RIF	n = 93	85.7% (48/56)	100%	3.2%	Active PTB	Adults	Pre-treated	2 cm^3	Uninfected	Xpert MTB/RIF	(71)
Xpert MTB/RIF	n = 267	62.1% (18/29)	99.6%	Not Stated	Culture-confirmed: per protocol	Children	Raw, stored in Sheather's solution	0.5 g	HIV infected	Xpert MTB/RIF	(80)
	n = 179	68.8% (11/16)	99.4%	None	Culture-confirmed: intention to diagnose	Children	Raw and stored	0.5 g		Xpert MTB/RIF	
Xpert MTB/RIF	n = 115	47.1% (8/17)	99.0%	None	Culture-confirmed	Children	Raw and stored	0.15 g	HIV uninfected and infected	Xpert MTB/RIF	(81)
Xpert MTB/RIF	n = 14	75.0% (3/4)	100%	None	Culture-confirmed	Children	Decontaminated	0.5 g	HIV uninfected and infected	Xpert MTB/RIF	(82)
Xpert MTB/RIF	n = 20	85.0%	100%	2.6%	TB cases	Children	Raw	0.6 g	HIV uninfected and infected	Xpert MTB/RIF	(83)
		84.0%	95.0%	7.8%	TB cases	Children	Raw	1.2 g		Xpert MTB/RIF	

*No volume specified only defined as “spoonful”

In recent studies, Walters *et al* (82) demonstrated a stool Xpert MTB/RIF sensitivity of 75% in a small subset of children compared to culture confirmed cases, following homogenisation and concentration of the specimens. Marcy *et al* (80) demonstrated sensitivities of 47.1% -68.8% using homogenised, gauze filtered and concentrated stool specimens for Xpert MTB/RIF analysis compared to culture confirmed cases in HIV-infected children. They also stated that larger volumes of stool specimens led to clogging of the Xpert MTB/RIF filters. Similarly Banada *et al* (83) concluded that a larger volume of homogenised, glass wool filtered stool (1.2 g) did not perform better than the smaller weight (0.6 g) and demonstrated a lower specificity (95%). Kokuto *et al* (71) demonstrated overall sensitivity of 85.7% with 100% specificity when testing pre-treated adult stool specimens on the Xpert MTB/RIF platform compared to active PTB cases. Their indeterminate rate was low at 3.2% (Table 1.2).

Stool specimens have been successfully used for the culture and molecular detection of MTB and show some potential as a diagnostic specimen. The sensitivities and specificities for the methods (Table 1.1 and Table 1.2) remain variable due to different stool preparation and processing methods, diverse patient populations and differences in the reference standard used. There is currently no gold standard method for processing of stool specimens for TB testing by culture or molecular methods. Given the limited data, all stool processing methods for culture need to be optimised for the detection of MTB from stool samples, and a balance between detection and contamination remains essential. Xpert MTB/RIF appears to perform better than heminested PCR assays to detect MTB DNA from stool specimens, particularly in children, but there is scope for improvement and optimization.

In public sector hospitals in South Africa, TB culture of stool specimens may be requested if abdominal TB is suspected. Stool specimens of >1 g may be sent to the TB laboratory when no other respiratory specimens can be obtained from a patient or if abdominal TB is suspected. An AFB stain is prepared from a portion of the stool specimen, and, if smear-positive, the original specimen is decontaminated using the routine NALC-NaOH Na-Citrate method (as is used on respiratory specimens) for liquid MGIT culture. If contaminated, the isolate will be re-decontaminated once and then discarded thereafter. Stool is currently not endorsed as a specimen type for the Xpert MTB/RIF assay and is thus not considered in the routine setting.

An alternative molecular detection method, the HAIN FluoroType assay, has been successfully used in adults using respiratory specimens. The FluoroType MTB test combines the use of specific primers and DNA amplification with melting curve analysis. The assay showed a sensitivity of 88.1% and a specificity of 98.9% compared to culture results with 0.7% of samples yielding invalid results (84) in a

recent study of adult patients. However, there are limited published data on the use of the automated FluoroType assay with stool specimens from children and adults (84).

1.5 Problem statement

The diagnosis of TB in children is problematic due to a combination of factors including the paucibacillary nature of disease associated with low smear, culture and Xpert sensitivities; the challenges in obtaining a representative specimen especially from children who cannot expectorate or from inaccessible extrapulmonary sites. A timely diagnosis remains essential due to rapid disease progression in children. As a result, treatment of children with possible TB is often started empirically before bacteriological confirmation is obtained. There is an urgent need to improve diagnostic algorithms, including faster and more accurate molecular confirmation methods with the addition of at least one first-line drug (preferably RIF) susceptibility profile. Accurate, rapid and cost-effective processing and testing methods for the detection of *M. tuberculosis* and associated drug-resistance from non-invasive stool specimens need to be established before stool can be adopted as a routine specimen type for paediatric TB diagnosis.

1.5.1 Aim

To evaluate the diagnostic utility of stool specimens using different protocols for the molecular detection of *Mycobacterium tuberculosis* in children investigated for suspected intrathoracic TB with or without extrathoracic TB.

1.5.2 Objectives

1) To determine the diagnostic performance of the Xpert MTB/RIF assay using:
    (a) decontaminated stool specimens; and
    (b) untreated raw stool specimens.

2) To determine the diagnostic performance of the Xpert MTB/RIF assay using a modified Xpert Tube Fill protocol on extracted DNA (FastDNA® Spin for Soil Kit (MP biomedicals)) from:
    (a) decontaminated stool specimens; and
    (b) untreated raw stool specimens.
3) To determine whether lyophilisation of stool specimens in combination with manual DNA extraction (QIAamp® DNA Stool Mini Kit (Qiagen)) enhances the diagnostic utility of the Xpert MTB/RIF assay using:
    (a) a modified Xpert Tube Fill protocol
    (b) the FluoroType (Hain Lifescience GmbH, Nehren, Germany) system
4) To determine whether lyophilisation of stool specimens in combination with an automated DNA extraction platform (GenoXtract (GXT, Hain Lifescience GmbH, Nehren, Germany)) enhances the diagnostic utility of the Xpert MTB/RIF assay using:
    (a) a modified Xpert Tube Fill protocol
    (b) the FluoroType system

CHAPTER TWO: Materials and methods

2.1 Study Setting and Laboratory Safety

This is a laboratory-based sub-study nested within a large prospective diagnostic cohort study entitled "Diagnostic yield and treatment response in childhood intra-thoracic tuberculosis: effect of disease severity" (Study PI: E. Walters). The prospective, hospital-based study was conducted in the South African National Accreditation System (SANAS) accredited (Appendix I) Biosafety Level 2 (BSL2) microbiology laboratory of the National Health Laboratory Service (NHLS) located at Tygerberg Hospital and the Division of Medical Physiology and Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences of the University of Stellenbosch, Tygerberg Campus. All positive cultures identified with genotypic resistance to rifampicin were sent to the SANAS accredited NHLS TB laboratory in Green Point (GP), Cape Town for second-line phenotypic susceptibly testing (ofloxacin and amikacin) using the MGIT indirect proportion method.

2.2 Study Population

The patient population included children younger than 13 years of age with and without HIV co-infection who were routinely evaluated for suspected intrathoracic (pulmonary) TB at the Tygerberg Children's and Karl Bremer Hospitals. Written consent for participation in the study was obtained from parents/legal guardians; in addition, assent was obtained from children ≥7 years of age, who demonstrated adequate understanding. Ethical approval for the study was given by the Health Research Ethics Committee, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa (Ethics reference number N11/09/282) (Appendix A).

2.3 Inclusion Criteria

Eligibility criteria for the clinical study included: Children less than 13 years of age, weighing more than 2.5 kg (as the study includes neonates) who were identified in hospital (inpatient or outpatient) with suspected intrathoracic TB (including suspected MDR-TB) based on persistent unremitting cough (or cough significantly worse than usual in a child with chronic lung disease including HIV-related) of >2

weeks duration and unresponsive to a course of appropriate antibiotics, poor growth documented over the preceding 3 months, persistent unexplained lethargy or reduced playfulness/activity reported by the caregiver, or unexplained fever >7 days. In addition, children <2 years of age were also eligible if they reported any duration of cough together with ≥1 of: a) documented exposure to a known TB source case (regardless of smear status), b) recent Mantoux TST conversion or reactive TST if not previously done or c) CXR suggestive of TB. In infants 0-60 days, additional inclusion criteria were unresponsive neonatal pneumonia or unexplained hepatosplenomegaly or sepsis-like illness.

2.4 Exclusion criteria

For the clinical study, children who had received >1 day of antituberculosis treatment before the first respiratory sample was collected, who had unstable clinical status as a contra-indication to intensive respiratory samples collection, who resided in a remote location, were excluded from the study. In addition, for this laboratory sub-study, participants who did not produce at least one stool specimen at enrolment were excluded.

2.5 Clinical investigation and specimen collection

Clinical investigations included a detailed history and examination, Mantoux TST, CXR, HIV test (HIV DNA PCR in children <18 months old, HIV ELISA in older children) and intensive specimen collection.

2.6 Respiratory specimen collection and transportation

Up to 6 respiratory specimens were collected over 2 days by trained nursing staff at participant enrolment inside a dedicated cough room in the hospital ward following infection control standards (Appendix L). GA and ES specimens were collected in 50 ml conical centrifuge tubes (LASEC SA) after a minimum 4 hour fasting period whilst IS and NPA specimens were collected in standard mucus extractor containers (25 ml, LASEC SA) after a minimum fast of 2 hours. Specimen pH was measured using disposable non-bleeding paper pH indicator sticks (Fisherbrand, Fisher Scientific, Suwanee, Ga, USA) and recorded in single pH units (pH 0-pH 14) using the supplied colour comparison chart. Acidic GA specimens were neutralised at the time of collection to pH 6-pH 7 by titrating small volumes of 4%

sodium bicarbonate solution (Appendix B). All specimens were placed in individual sealable transport bags. Specimen containers were labelled with unique barcoded identifiers and were accompanied by specimen specific documentation which was placed in a separate outer pocket of the transport bag. All containers and transport bags were disinfected (alcohol, 70%) before transportation in a transport box containing ice bricks (2-8°C) to the microbiology laboratory of the NHLS at Tygerberg Hospital. Specimens were refrigerated (2-8°C) until processing within 3 days of collection. Transport temperatures were recorded on study specific forms (Appendix J) and fridge temperatures were monitored by an electronic monitoring system.

2.7 Respiratory specimen processing

Appropriate personal protective equipment and clothing were used at all times when handling and processing specimens. Workbench and cabinet surfaces were cleaned and sterilized before and after specimen handling and all biohazardous waste was properly disposed of (85). Reagents were freshly prepared, replaced regularly and only one specimen container was open at any given time to limit cross contamination.

2.7.1 Digestion and decontamination

The labelled specimens were received and all patient plus specimen identification labels were captured onto the NHLS Laboratory Information System (LIS; DISA/Trakcare). Specimens were removed from the transport bag inside the biosafety cabinet and any leaked specimens were properly discarded and all relevant specimen information was recorded. Specimens were transferred to a sterile 50 ml conical centrifuge tube (except for specimens already received in a conical tube). For specimens, less than 3 ml in volume, 0.67 M PBS (pH 6.8; NHLS GP, South Africa) was added to a total volume of 3 ml (visually estimated using a pre-labelled 50 ml conical centrifuge tube for volumes less than the calibrated 5 ml). To each specimen, an equal volume of NALC-NaOH-sodium citrate (100 ml solution: 5.0% NaOH solution mixed 1:1 with 2.9% sodium citrate solution, and 0.5 g NALC) was added prior to vortex mixing and incubation for 17 minutes at room temperature (41). Specimens were neutralised by the addition of PBS up to the 40 ml mark on the conical container followed by centrifugation at 3000xg at 4°C for 20 minutes. The supernatants were decanted into a liquid waste

bottle and the sediments resuspended in approximately 3 ml PBS (decontaminated specimen suspension) (40).

2.7.2 Concentrated smear microscopy

One drop of decontaminated specimen suspension was mixed with a drop of TB precipitating fluid (NHLS, GP) on a labelled microscope slide creating a smear size of between 1.5 and 2.0 cm^2. The smears were dried on a heating block at 70°C for 2 hours before staining with Auramine O solution (NHLS). Auramine smears were fixed with methanol for 30 seconds, flooded with Auramine O stain and incubated at room temperature for 20 minutes. Excess Auramine O stain was rinsed off with tap water and the smear was decolourised with 0.5% Acid Alcohol for approximately 2 minutes followed by rinsing. The smears were flooded with potassium permanganate counter stain for 2 minutes followed by rinsing and air-drying. Acid fast organisms emitted a bright yellow fluorescence when viewed under a fluorescent microscope. At least 100 fields were viewed (at 40x magnification) per smear and results were reported as negative, scanty, 1+, 2+ or 3+ according to the WHO/IUATLD standardised guidelines (40). Ten percent of all negative and all positive smears (including positive (H37Rv laboratory strain) and negative control slides) were checked by a second reader daily.

2.7.3 Liquid culture

From the decontaminated specimen suspension, 0.5 ml was inoculated into a 7 ml MGIT containing 0.8 ml BBL MGIT PANTA/OADC mixture (Becton Dickson; Appendix B) followed by incubation in the Bactec MGIT 960 system at 37°C. Bacterial growth was monitored hourly on the Bactec MGIT 960 automated liquid culture system. Once the MGIT tubes were in the Bactec system, they were incubated for a maximum of 42 days. If no significant change in fluorescence was automatically detected by the system within 42 days, the culture was reported as being negative for MTB. Negative cultures were manually checked for any visible growth after the incubation period before being discarded. If any visible growth was observed the specimen would be processed further as a positive culture for further identification. Cultures reported as positive by the Bactec system, or where visible growth was observed, were batched and processed weekly for further identification and DST. The Bactec system also records the TTD for each positive culture. Daily negative (PBS), and weekly positive (H37Rv laboratory reference strain) controls were included. All reagents are checked before use to

enforce expiration dates and batch verification tests (H37Rv serial dilutions) for MGIT tubes are done on receipt.

2.7.4 Xpert MTB/RIF Assay

Specimen testing on the Xpert MTB/RIF Assay was done as per manufacture's instruction. Briefly, 1 ml of the decontaminated specimen suspension was mixed with 2 ml Xpert MTB/RIF sample reagent (1:2 ratio) in a sterile 50 ml conical tube. This mixture was incubated at for 10 minutes at room temperature, briefly mixed by inverting the tube 10 times and incubated for a further 5 minutes. The mixture was completely transferred to a pre-labelled Xpert MTB/RIF cartridge and analysed in the GeneXpert® instrument (GeneXpert Dx software version 4.4a). If an "invalid", "error" or "No Result" result (Table 2.1) occurred; and if sufficient decontaminated specimen suspension remained (1 ml), the Xpert MTB/RIF assay was repeated once.

Table 2.1. Xpert MTB/RIF assay result reporting

MTBC	Semi Quantitative Readout	RIF Resistance
NO RESULT	N/A	N/A
ERROR	N/A	N/A
INVALID	N/A	N/A
MTB NOT DETECTED	N/A	N/A
MTB DETECTED	Very LOW	INDETERMINATE, NOT DETECTED or DETECTED
	LOW	
	MEDIUM	
	HIGH	

2.7.5 Ziehl-Neelsen (ZN) microscopy

For all positive MGIT cultures, one drop of the resuspended culture was added to a drop of TB precipitating fluid (NHLS) on a labelled microscope slide and dried on a hot plate for at least 2 hours at 70°C. All staining steps were performed inside a fume hood (NHLS). Microscope slides for ZN staining were flooded with carbol fuchsin stain (NHLS) and gently heated for approximately 1 minute

with an open flame until steam arose, melting the outer wax layer of any AFB. The smears were incubated at room temperature for 5 minutes and excess carbol fuchsin stain was rinsed off with tap water. The smears were decolourised with acid alcohol (3%; NHLS) for approximately 2 minutes followed by rinsing, with tap water. Slides were then flooded with methylene blue (0.3%; NHLS) counter stain for approximately 2 minutes followed by rinsing and air-drying. Acid-fast organisms appeared as reddish rod-shaped structures often forming cord-like structures when viewed under a light microscope.

2.7.6 Blood Agar Plates (BAP)

All positive MGIT cultures were plated out on BAP and incubated at 35°C for at least 24 hours to detect any contamination. BAP results were recorded as “growth” or “no growth”. The combination of ZN microscopy and BAP was used as a purity check to confirm the presence of AFB and to rule out contaminated cultures. Positive MGIT cultures that displayed acid-fastness after ZN staining along with no contaminant growth on the BAP had further identification and DST done (Appendix G). Contaminated cultures that displayed acid-fastness were re-decontaminated once more to obtain a pure culture for DST. Repeat decontamination was done by adding the contents of the MGIT tube to a sterile 50 ml conical tube with the addition of an equal volume of 4% NaOH, followed by incubation for 17 minutes at room temperature. The specimen was then processed further as per Section 2.7.1 and re-incubated (up to 42 days). Continued contaminated cultures not displaying acid-fastness were recorded as “contaminated” and no further testing was done.

2.7.7 HAIN GenoType®MTBDR*plus* Assay

Genomic DNA was extracted from positive MGIT cultures using the GenoLyse method (86) as per the manufacturer’s instructions (HAIN GenoLyse VER 1.0, Hain Lifescience GmbH, Nehren, Germany). Briefly, 1 ml of the positive MGIT liquid media was transferred to a pre-labelled sterile 1.5 ml plastic screw cap tube and centrifuged for 15 minutes at 10 000xg. Negative (sterile water) and positive (H37Rv laboratory reference strain) controls were included in each batch. The supernatants were discarded and sediments resuspended in 100 µl lysis buffer (A-LYS) by vortex mixing. Aliquots were heat inactivated at 95°C for 5 minutes and sonicated for 15 minutes followed by the addition of 100 µl neutralisation buffer (A-NB) and vortex mixing for 5 seconds. The specimens were centrifuged for 5

minutes at 10 000xg and 5 µl of the supernatant was transferred to a PCR tube for DNA amplification and the remainder was transferred to a sterile 1.5 ml plastic screw cap tube for short term storage at 2-8°C if repeat testing was required.

The GenoType®MTBDR*plus* test was performed for genotypic identification of MTBC and drug sensitivities to RIF and INH as per the manufacturer's instruction (MTBDR*plus* VER 2.0, Hain Lifescience GmbH, Nehren, Germany). Resistance to RIF was identified by detection of the most significant *rpo*B gene mutations. High level INH resistance was identified by the detection of mutations in the *kat*G gene, whilst low level INH resistance was identified by mutations in the *inh*A promoter region.

The DNA amplification mix (AM) was prepared as follows: Stored amplification reagents, AM-A and AM-B (-20°C) were thawed to room temperature, and 10 µl AM-A and 35 µl AM-B were added to each 5 µl of extracted DNA specimen (final reaction mix volume of a 50 µl). To limit the risk of cross contamination the DNA extraction, PCR mix preparation, DNA addition and hybridisation steps were conducted in separate rooms. The PCR amplification conditions were as follows: initial denaturation at 95°C for 15 minutes, 10 cycles of denaturation at 95°C for 30 seconds, 65°C for 2 minutes, 20 cycles of denaturation at 95°C for 25 seconds with annealing at 50°C for 40 seconds, elongation at 70°C for 40 seconds and final extension at 70°C for 8 minutes (standard ramp rates). Following PCR amplification, the chemically denatured amplicons (single stranded) were hybridised (reverse hybridisation) to the membrane strips (coated with specific probes complimentary to the amplified nucleic acids) in the TwinCubator according to the manufacturer's instructions. Briefly, 20 µl of biotin labelled amplicon was hybridised onto the strip with 1 ml hybridisation buffer. After stringent buffer washing, the streptavidin-alkaline-phosphatase conjugate was added to the strip. An alkaline phosphatase mediated staining reaction occurred where the probe and amplicon hybridised, resulting in the appearance of a dark banded pattern. Hybridisation patterns were interpreted according to the manufacturer's guidelines (HAIN MTBDR*plus* VER 2.0). Results were reported as MTB negative or MTB positive with respective resistance profiles when applicable (Table 2.2). Cultures suspected of being NTM (ZN positive without characteristic cording pattern and negative by the MPT64 assay) were tested with the GenoType Mycobacterium CM/AS kit as per manufacturer's instruction (Hain Lifescience GmbH, Nehren, Germany). RIF resistant cultures were submitted to the NHLS GP laboratory for 2nd line susceptibility testing by the agar indirect proportion method (critical concentrations: ofloxacin (2.0 µg/ml and amikacin 4.0 µg/ml).

Table 2.2. Summary of resistance profiles and further testing

MTBC (MTBDR*plus*)	RIF	INH	Second-line DST (NHLS - GP)	NTM ID (Mycobacterium CM/AS)
Not detected	N/A	N/A	N/A	YES
Detected	Susceptible	Susceptible	NO	NO
Detected	Resistant	Susceptible	YES	NO
Detected	Susceptible	Resistant	YES	NO
Detected	Resistant	Resistant	YES	NO

2.8 Stool Specimen collection and transportation

During the initial phase of this study, stool specimens obtained between April 2013 and June 2014 were collected by trained nursing staff in sterile containers (25 ml Fecal Cup With Spoon, LASEC SA) with an equal volume of sterile saline added to each specimen by the nursing staff at the time of collection. Specimens were homogenised by inverting the tube 10-15 times by hand and transported to the lab to be stored at (2-8°C) until further analysis by MGIT culture, concentrated smear microscopy and Xpert MTB/RIF assay testing (Method A) as summarised in Table 2.3.

Table 2.3. Stool study protocol: Method A

Stool	Quantity	Use
1 portion only	0.3 g to 5.0 g	Concentrated smear microscopy, MGIT culture and Xpert MTB/RIF analysis (Method A) Decontaminated remainder stored in PBS at -20°C

Stool specimens processed using this method (Method A) were also tested by MGIT culture. However, due to a high proportion of contaminated stool cultures (41.5%; Walters E, unpublished data), stool culture was discontinued in June 2014. After June 2014, raw stool specimens were collected for direct Xpert MTB/RIF investigation, without the addition of sterile saline (Method B, Table 2.4). Stool specimens were collected from each participant at the baseline visit and these were split into two stool portions (portion 1 and portion 2) in separate containers (25 ml Fecal Cup With Spoon, LASEC

SA) by trained nursing staff. The first portion was analysed by direct Xpert MTB/RIF assay (Method B) and the second portion was stored at -20°C until lyophilisation.

Table 2.4. Stool study protocol: Method B

Stool	Quantity	Use
Portion 1	0.3 g to 5.0 g	Direct Xpert MTB/RIF analysis (Method B)
Portion 2	0.3 g to 5.0 g	Raw stool stored at -20°C

2.9 Stool specimen processing

Stool specimens were processed according to the study specific protocols (Method A and Method B) and the remainder from Method A and portion 2 from Method B were stored at -20°C for further analysis.

2.9.1 Method A - Stool specimen decontamination, concentration and MTB detection using smear, culture and the Xpert MTB/RIF assay

Stool specimens in saline buffer were received in the lab and 5-10 ml PBS buffer was added to each container. The specimens were homogenised by vortex mixing for 20 seconds followed by incubation at room temperature for 5 minutes allowing the settling of large debris particulates. Five millilitres of this homogenised stool supernatant was transferred to a labelled 50 ml conical centrifuge tube. An equal volume of NALC-NaOH-sodium citrate was added followed by vortex mixing and incubation for 17 minutes at room temperature. Specimens were neutralised by the addition of PBS up to the 40 ml mark on the conical container followed by centrifugation at 3000xg at 4°C for 20 minutes. The supernatants were decanted into a liquid waste bottle and the sediments resuspended in approximately 3 ml PBS (decontamination protocol, section 2.7.1). The decontaminated resuspended stool sediments were analysed by concentrated smear microscopy (Auramine), liquid (MGIT) culture and Xpert MTB/RIF Assay as previously described for respiratory specimens in section 2.7 (one drop for smear microscopy, 0.5 ml for liquid culture and 1 ml for Xpert) and the remainder of this resuspended sediment was stored at -20°C (Figure 2.1).

2.9.2 Method B - Stool specimen concentration and MTB detection using direct Xpert MTB/RIF detection

A large scoop (0.3 g-5.0 g) of portion 1 stool specimen was added to a 50 ml conical centrifuge tube. The stool specimens were homogenised in 10 ml PBS buffer by vortex mixing for approximately 20 seconds (or until completely homogenised) followed by centrifugation at 3000xg for 20 minutes at 4°C. The supernatants were discarded and the sediments resuspended in up to 10 ml PBS buffer by vortex mixing for approximately 20 seconds. The resuspended sediments were centrifuged briefly (1 second at 2000xg) to settle large debris particulates. One millilitre of the supernatant was aspirated from the bottom layer (above sediment) and used for direct Xpert MTB/RIF analysis as per section 2.7.7 (Figure 2.1). If an indeterminate result occurred, the Xpert MTB/RIF assay was repeated once per stool specimen using the remaining supernatant from the brief centrifugation step.

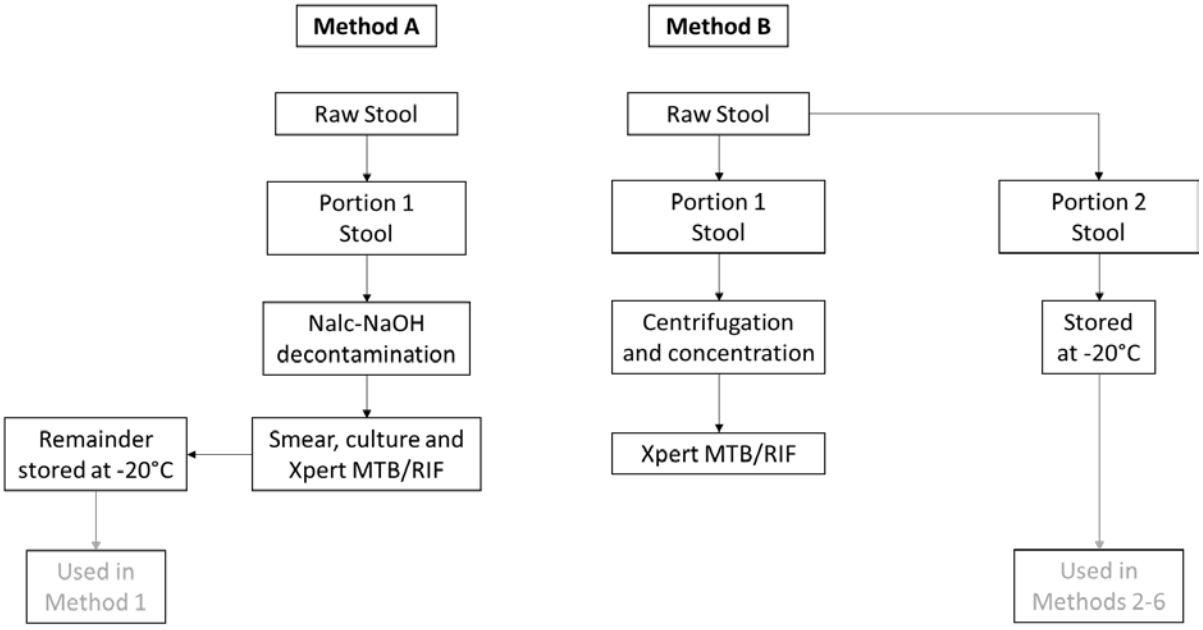

Figure 2.1. Overview of stool processing Methods A and B

2.9.3 Lyophilisation

The decontaminated stool suspension (remainder from Method A) was not lyophilised but subjected to Method 1 (section 2.10.1), whereas portion 2 (raw) stool, stored from stool specimens collected and tested by Method B, was either thawed and used for Method 2 or subjected to the lyophilisation process and subsequently tested using Methods 3-6 (section 2.10.3 - 2.10.6). Stored raw stool specimens (-20°C) were thawed at room temperature and transferred to a sterile 50 ml conical centrifuge tube. The mass of each specimen, pre-lyophilisation and post-lyophilisation, was recorded. One hundred microliters of sterile 10x Tris-HCL (10 mM)/EDTA (10 mM) pH 8 (TE) buffer (Appendix B) was added to each specimen to preserve the DNA. The specimens were heat inactivated at 80°C for 1 hour in a water bath (Memmert, LASEC SA). Heat inactivated stool specimens were lyophilised prior to DNA extraction to allow standardisation of input specimen mass.

The prepared heat inactivated specimens were sealed with a layer of parafilm to prevent cross-contamination during lyophilisation. Specimens were placed in a metal tray; small openings in the parafilm were made using a 1 ml sterile tuberculin syringe (LASEC SA) to allow for moisture escape and the specimen tray was submerged in liquid nitrogen for approximately 30 seconds (flash freezing). The frozen specimens were placed in the vacuum chamber of the Labconco freeze dryer (Vacutec, South Africa). The sealed chamber was left to reach a vacuum at 37 Pascals at -49°C and specimens were freeze-dried for 24 hours. Specimens that were not completely dry (visually assessed) were lyophilised for an additional 24 hours.

2.9.4 Manual DNA extraction protocols

2.9.4.1 FastDNA® Spin for Soil Kit (MP biomedicals)

The stored decontaminated stool suspension (remainder from method A, Section 2.9.1) or the stored raw stool (Portion 2, remainder from Method B) was thawed at room temperature and 100 µl was transferred to a lysing matrix E tube (used in Method 1 and Method 2 respectively). Genomic DNA was extracted using the FastDNA® Spin for Soil Kit as per the manufacturer's instructions (Appendix C) from these stool specimens. The DNA was eluted in 200 µl elution buffer instead of the recommended 100 µl to accommodate the recommended starting volume of the Xpert Tube Fill protocol (Section 2.9.6.1).

2.9.4.2 QIAamp® DNA Stool Mini Kit

The QIAamp DNA Stool Mini Kit extraction protocol was performed from lyophilised specimens (Sections 2.10.3, 2.10.4; and 2.9.3). Genomic DNA was extracted from the lyophilised stool specimens according to the manufacturer's instructions (Appendix D) with some protocol modifications, namely: The starting amount for each lyophilised stool specimen was kept constant at 100 mg (+- 5 mg) instead of the recommended 180-220 mg of raw stool for consistency. The lysis temperature was increased from 70°C to 95°C for difficult to lyse cells (Gram-positive bacteria). Purified genomic DNA was eluted in 200 µl elution buffer to accommodate the Xpert Tube Fill protocol (Section 2.9.6.1.).

2.9.5 Automated Hain GenoXtract DNA extraction protocol

Automated DNA extraction was done from lyophilised stool specimens using the GenoXtract along with the GenoXtract DNA/RNA Extraction Kit as per the manufacturer's instructions (Appendix E). Briefly, lyophilised specimens were swabbed (approx. 100 mg) and reconstituted in 100 ml of stool stabiliser. Pump-pipette-tip units, reagent cartridges and elution containers were mounted in the GenoXtract equipment. The GenoXtract software was set to elute the isolated DNA into 200 µl elution buffer following the 40-minute protocol.

2.9.6 Molecular detection of MTB

2.9.6.1 Xpert Tube Fill protocol

Extracted genomic DNA was tested on the Xpert MTB/RIF using a Tube Fill protocol and software adaptation. In brief, the cartridge lid was opened and 200 µl of purified DNA was transferred to the 6th well of an Xpert MTB/RIF cartridge (Figure 2.2) using a sterile 1 ml syringe (Avacare, LASEC SA) and a spinal needle (21GX1'' 0.8x25 mm TW, Avacare, LASEC SA). The DNA mixture was added close to the bottom of the well (approximately 1 mm from the bottom) ensuring that no air bubbles were present

and without piercing the container. This method bypasses the initial chambers responsible for specimen washing and DNA extraction.

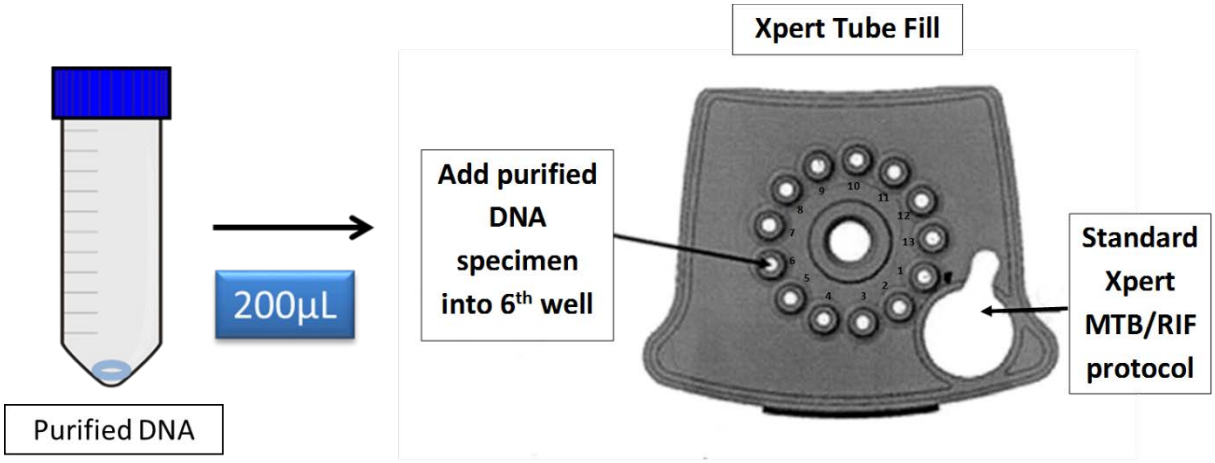

Figure 2.2. Schematic representation of Xpert Tube Fill protocol

The cartridges were analysed using the GeneXpert instrument with a preloaded Tube Fill (ADF file) software add-on. Once the cartridge information was manually entered, by following the software prompts, the specific MTB Tube Fill (version 1.0) assay was selected from the dropdown list. The cartridges were placed in the module and the automated system generated results within 1 hour 35 minutes.

2.9.6.2 Fluorotype MTB protocol (Hain Lifescience GmbH, Nehren, Germany)

The Fluorotype MTB test was done on the FluoroCycler 12 instrument as per the manufacturer's instructions (Appendix F). Briefly, PCR reaction mixtures were freshly prepared by combining 3 µl amplification mix A (AM-A) with 7 µl of amplification mix B (AM-B) in 200 µl PCR reaction tubes (Sarstedt, Nürnberg Germany). Six microliters of the GenoXtract extracted DNA solution was added to 10 µl of amplification mix. Kit controls included 6 µl of sterile PCR-grade water (non-template control) and 6 µl of C + FT MTB (positive control). The PCR mixtures were loaded onto the FluoroCycler instrument with the accompanying automated analysis FluoroSoftware IVD 1.1.0. The melting curve for the amplification control was expected at 70.5°C (+/- 3°C) and a positive MTBC result was expected

at 60.0°C (+/- 3°C). Analysis software expressed results as either: “no MTB complex DNA detected”; “MTB complex DNA detected”; “Not interpretable”; or “Invalid” (Table 2.5).

Table 2.5. FluoroType result interpretation

Software Interpretation	Explanation
MTB complex DNA detected	There is a peak at the MTB complex-specific melting point
No MTB complex DNA detected	There is no peak at the MTB complex-specific melting point
Negative control valid	As expected, no peak at the MTB complex-specific melting point
Positive control valid	As expected, there is a peak at the MTB complex-specific melting point
Not interpretable	Peaks detected outside of the control or MTBC-specific melting point
	Additional peaks detected
Invalid	No valid peaks detected
	Discordant control results

2.10 Stool specimen molecular detection protocols

In an attempt to improve the sensitivity of Xpert on stool specimens for the bacteriological confirmation of TB in children, combinations of DNA extraction methods and molecular detection platforms (Methods 1 – 6, Figure 2.3) were used.

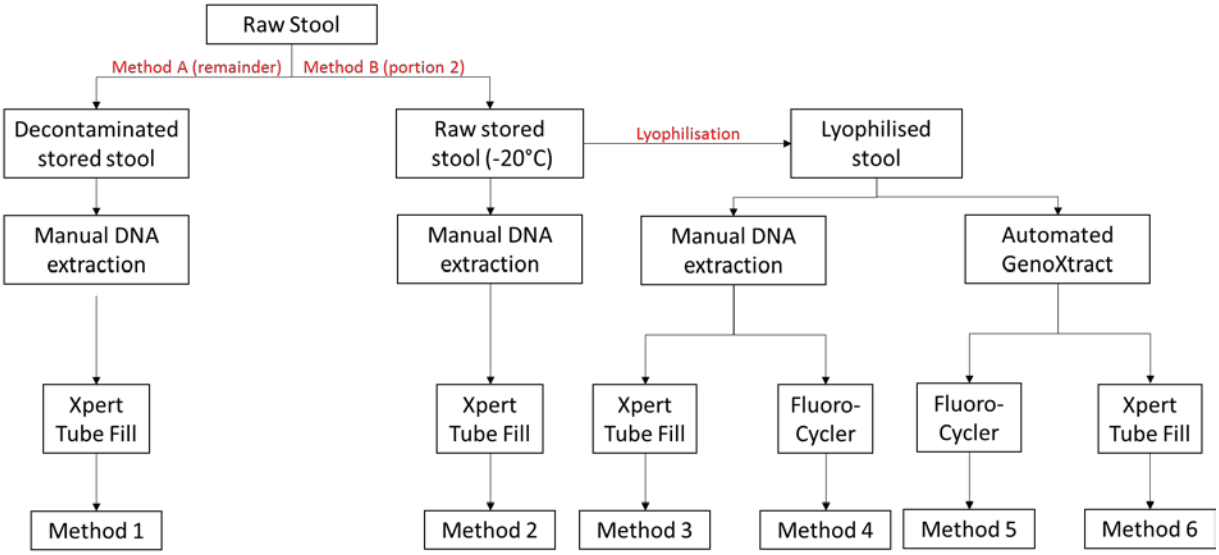

Figure 2.3. Overview of stool processing methods 1 - 6

2.10.1 Method 1 – FastDNA DNA extraction followed by the Xpert Tube Fill protocol on decontaminated stool specimens

Genomic DNA was extracted from the stored remainder of the decontaminated stool suspension of method A (Section 2.9.1) using the FastDNA® Spin for Soil Kit as per section 2.9.4.1. Molecular detection was completed using the Xpert Tube Fill platform (Section 2.9.6.1).

2.10.2 Method 2 – FastDNA DNA extraction followed by the Xpert Tube Fill protocol on untreated raw stool specimens

When Method A was discontinued, stool specimens were no longer stored in the post-decontamination fluid (remaining NALC-NaOH-sodium citrate-PBS buffer) and subsequently DNA extraction was completed on raw stool specimens (portion 2 specimens, Method B) using the FastDNA® Spin for Soil Kit as described above (Section 2.10.1). Molecular detection was completed using the Xpert Tube Fill protocol (Section 2.9.6.1).

2.10.3 Method 3 – QIAamp DNA extraction followed by the Xpert Tube Fill protocol on lyophilised stool specimens

A major challenge for the molecular processing of stool specimens was the variability in stool consistencies. A reproducible starting mass could not be consistently obtained for the DNA protocols. It was decided that the removal of water from the stool specimens by lyophilisation would allow for an easy-to-work-with specimen and potentially concentrate any intact or extracellular MTB present in the specimen.

Stored raw stool specimens were lyophilised (Section 2.9.3) prior to manual DNA extraction using the QIAamp® DNA Stool Mini Kit (Section 2.9.4.2). Due to availability and cost limitations, the FastDNA® Spin for Soil Kit was replaced with the QIAamp® DNA Stool Mini Kit (serial dilutions using H37Rv yielded similar results for both kits).

2.10.4 Method 4 – QIAamp DNA extraction followed by the FluoroType protocol on lyophilised stool specimens

The HAIN FluoroType protocol was considered as a detection assay as it has the ability to test multiple specimens simultaneously. DNA was extracted from lyophilised stool using the QIAamp® DNA Stool Mini Kit (Section 2.9.4.2) and molecular detection was done using the HAIN FluoroType protocol (Section 2.9.6.2).

2.10.5 Method 5 – GenoXtract DNA extraction followed by the FluoroType protocol on lyophilised stool specimens

The automated protocol of extracting numerous specimens simultaneously seemed more feasible than the hands-on and more labour-intensive manual DNA extraction protocols. DNA was extracted from lyophilised stool using the automated HAIN GenoXtract (Section 2.9.5) and molecular detection was done on the using the HAIN FluoroType platform (Section 2.9.6.2).

2.10.6 Method 6 – GenoXtract DNA extraction followed by the Xpert Tube Fill protocol on lyophilised stool specimens

To compare the two detection platforms, DNA extracted using the automated HAIN GenoXtract was tested on the Xpert Tube Fill detection platform.

Table 2.6. Summary of stool processing methods

Method	Description	Stool processing	Extraction	Amplification	Detection
A	Decontaminated Standard A	Raw + Decontaminated	(Xpert)*	(Xpert)*	Xpert
B	Direct Raw Standard B	Raw + PBS middle layer	(Xpert)*	(Xpert)*	Xpert
1	Decontaminated Remainder from Method A	Raw + Decontaminated + PBS (Stored -20°C)	Soil kit	(Tube Fill)**	Tube Fill
2	Manual Extraction/ Tube Fill	Raw (Stored -20°C)	Soil kit	(Tube Fill)**	Tube Fill
3	Manual Extraction/ Tube Fill	Lyophilised	Qiagen kit	(Tube Fill)**	Tube Fill
4	Manual Extraction/ FluoroType	Lyophilised	Qiagen kit	FluoroType	FluoroType
5	Automated Extraction/ FluoroType	Lyophilised	GenoXtract	FluoroType	FluoroType
6	Automated Extraction/ Tube Fill	Lyophilised	GenoXtract	(Tube Fill)**	Tube Fill

* DNA extraction and amplification occurs within the Xpert cartridge

** DNA amplification occurs within the Xpert cartridge

2.10.7 Proof-of-concept protocol

A dilution range (undiluted to 10^{-7}) was made of purified H37Rv DNA (laboratory reference strain, 25 ng/µl) to determine the detection limit of the Xpert Tube Fill and FluoroCycler protocols and to serve as a proof-of-concept for the recovery of MTB DNA from spiked stool specimens. This was done for Methods 1,2,3,5 and 6. Method 4 did not have spiking experiments done. The number of molecules (N) per mole present in the H37Rv DNA dilution range was calculated according to the following equation:

$$N = nNa$$

$$= \left(\frac{C}{Mw}\right) Na$$

Na = the Avogadro constant = 6.023 x10^{23}

C= initial DNA concentration

n= number of mole = C/Mw

Mw= number of base pairs in *MTB* genome (4.4 x10^{6}) x average molecular weight of a base pair (700)

The GeneXpert targets the *rpoB* gene which is present in single copy in the MTB genome, therefore the dilution range represented 0 to ~5 million bacteria per µl (Table 2.7).

Table 2.7. Number of molecules in each pure H37Rv DNA dilution

Sample Dilution	Sample Concentration (ng/µl)	Number of molecules/µl
(10^{0})	25	4888799
(10^{-1})	2.5	488880
(10^{-2})	0.25	48888
(10^{-3})	0.025	4889
(10^{-4})	0.0025	489
(10^{-5})	0.00025	49
(10^{-6})	0.000025	5
(10^{-7})	0.0000025	≤1

2.11 Analysis plan and definitions

The primary reference standard for diagnostic accuracy calculations was a composite reference standard defined as respiratory mycobacteriology. This composite reference standard included any MGIT culture and/or Xpert MTB/RIF results from any respiratory specimens collected for the study. The reference was considered positive if any of these results were positive, and negative if all of the results were negative. Although false positive results on the reference standard are always a possibility, the study population consisted of children with a high pre-test probability of having active TB disease. Given the high positive likelihood ratios for both MGIT culture and Xpert MTB/RIF, the

post-test probability of active TB for children with positive test results is very high. Furthermore, recently developed consensus case definitions for PTB in children classify as a case of confirmed TB any child presenting with symptoms, and with MTB identified by mycobacterial culture or Xpert MTB/RIF on any respiratory specimens (87).

Due to possible bias resulting from the absolute number of respiratory specimens processed (up to 6) compared to only one stool specimen tested on Xpert MTB/RIF, Xpert Tube Fill and/or the HAIN FluoroType assay, a secondary reference standard was defined as the "first respiratory specimen" collected from any participant for the study, as one sample better reflects the routine practice in our setting and is a fairer comparison. All ZN negative and contaminated (after repeat decontamination) and NTM culture results were excluded from analyses and "Indeterminate" results were defined as "error", "invalid" or "no result" for Xpert MTB/RIF, "error" only for Tube Fill assays, and as "not interpretable" or "invalid" for the FluoroType assay. Indeterminate rate was defined as the number of indeterminate results over the number of stool specimens tested for the composite reference standard, and was calculated separately for each individual method. All indeterminate results and any expected tests that were not done for any reason were omitted from sensitivity and specificity calculations.

CHAPTER THREE: Results

3.1 Stool processing from study specific protocols (Method A and B)

A total of 517 children were enrolled of whom 405 provided a stool sample for analysis, 109 participants did not provide a stool specimen and 3 participants withdrew from the study before any specimens were collected (Figure 3.1).

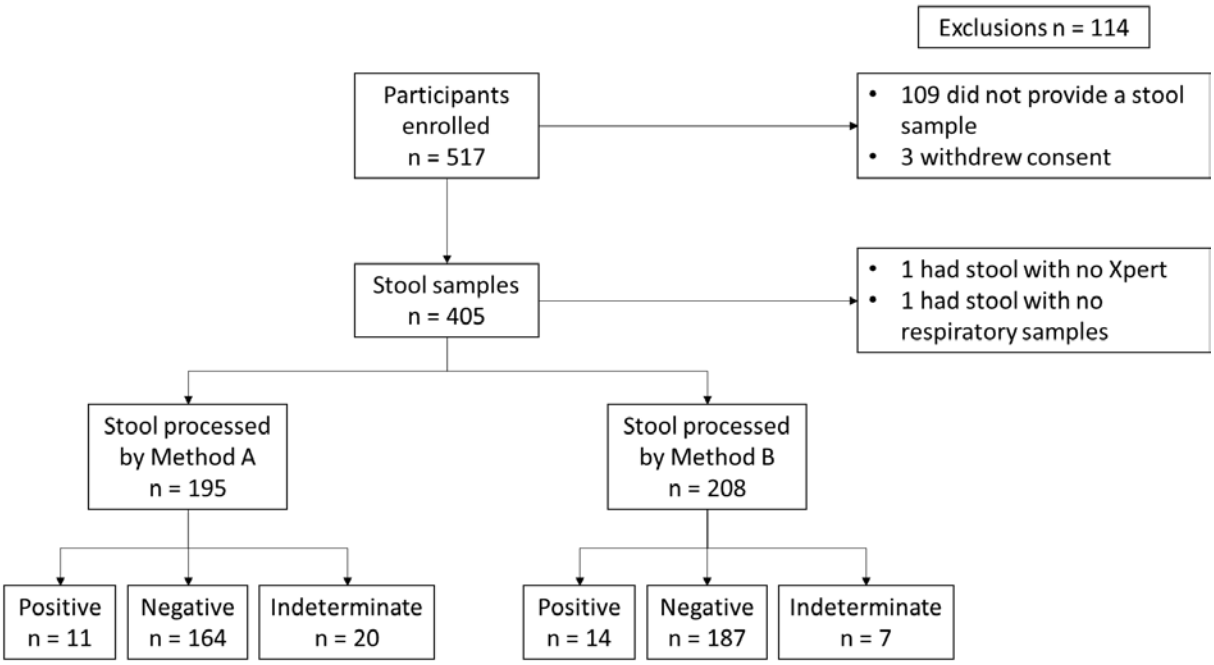

Figure 3.1. Overview of Stool results processed by Method A and B

3.1.1 Method A - Stool specimen decontamination, concentration and MTB detection using Xpert MTB/RIF assay

A total of 195 participant stool specimens were processed by Method A. The sensitivity and specificity (Excel developed calculation based on Chi-squared analysis) of stool Xpert compared to the composite reference standard were 26.7% and 97.9% (n = 195), while the performance improved to 47.1% and 97.8%, respectively, compared to the first respiratory specimen culture (n = 166) and to 75.0% and 96.3% compared to the first respiratory Xpert (n = 96) (Table 3.1). During the initial phases of the study many Xpert MTB/RIF assays were not done on the first respiratory specimen as they formed part of a

study-specific protocol where different specimen types were combined for Xpert MTB/RIF detection. These specimens were excluded from this analysis. The positive predictive value (PPV) was the highest for the composite reference standard and the first respiratory culture at 72.7% and the probability decreased for the first respiratory Xpert to 66.7%. The negative predictive value (NPV) was the lowest for the composite reference standard at 86.6% and increased to 93.6% and 97.5% for the first respiratory culture and Xpert respectively. The overall indeterminate rate was 20/195 (10.3%).

Table 3.1. Method A Results summary

Method A: Stool Decontamination + Xpert	**Composite Reference Standard**		**First Respiratory Culture**		**First Respiratory Xpert**	
Result	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE
POSITIVE	8	3	8	3	6	3
NEGATIVE	22	142	9	132	2	77
INDETERMINATE	4	16	1	13	1	5
TOTAL	**34**	**161**	**18**	**148**	**9**	**85**
Sensitivity	**26.7**		**47.1**		**75.0**	
Specificity	**97.9**		**97.8**		**96.3**	

3.1.2 Method B - Stool specimen concentration and MTB detection using direct Xpert MTB/RIF detection

208 participant stool specimens were processed by Method B for Xpert MTB/RIF analysis. The sensitivity and specificity of stool Xpert compared to the composite reference standard were 40.0% and 100% (n = 208), 66.7% and 97.7% compared to the first respiratory specimen culture (n = 198) and 45.8% and 98.2% compared the first respiratory Xpert (n = 196), respectively. The PPV was the highest for the composite reference standard at 100.0% and decreased for the first respiratory culture to 78.6% and to 71.4% for the first respiratory Xpert. The NPV was the lowest for the composite reference standard at 88.8% and increased to 92.6% and 97.2% for the first respiratory culture and Xpert respectively. The indeterminate rate was 7/208 (3.4%).

Table 3.2. Method B Results summary

Method B: untreated stool Xpert	**Composite Reference Standard**		**First Respiratory Culture**		**First Respiratory Xpert**	
Result	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE
POSITIVE	14	0	11	3	10	4
NEGATIVE	21	166	13	163	5	173
INDETERMINATE	2	5	2	4	1	5
TOTAL	**37**	**171**	**26**	**170**	**16**	**182**
Sensitivity	**40.0**		**45.8**		**66.7**	
Specificity	**100.0**		**98.2**		**97.7**	

3.1.3 Proof-of-concept protocol experiment for DNA extraction protocols

As a proof-of-concept experiment, dilutions of MTB (H37Rv laboratory reference strain) of known concentration were used to determine the extraction efficiency of the various DNA extraction and molecular detection protocols from DNA inoculated stool specimens. Xpert Tube Fill detection from lyophilised stool processed by manual DNA extraction methods (both soil and stool kits) detected MTB DNA between 0.25 fg/µl and 2.5 fg/µl, equivalent to 0 – 5 bacteria per µl (Table 3.3). The FluoroType assay showed the same extraction efficiency using lyophilised stool with automated GenoXtract. The extraction efficiency for MTB DNA extracted from lyophilised stool using the GenoXtract when detected on the Xpert Tube Fill assay was however lower at 0.25 pg/µl to 2.5 pg/µl (49 – 490 bacteria per µl). MTB DNA was detected at the upper limit or highest DNA concentration of 25 ng/µl (approx. 5 million bacteria per µl) on all methods, showing that a high concentration of DNA does not interfere with the detection assays.

Table 3.3. Proof-of-concept results for MTB detection

Sample Dilution	Number of molecules/ µl	Direct DNA; NO stool	Method 1 (2.10.1)	Method 2 (2.10.2) and Method 3 (2.10.3)	Method 5 (2.10.5)	Method 6 (2.10.6)
(10^0)	4888799	Detected	NOT DONE*	Detected	Detected	Detected
(10^{-1})	488880	Detected	Detected	Detected	Detected	Detected
(10^{-2})	48888	Detected	Detected	Detected	Not Detected**	Not Detected**
(10^{-3})	4889	Detected	Detected	Detected	Detected	Detected
(10^{-4})	489	Detected	Detected	Detected	Detected	Detected
(10^{-5})	49	Detected	Detected	Detected	Detected	Not Detected
(10^{-6})	5	Detected	Not Detected	Detected	Detected	Not Detected
(10^{-7})	≤1	Detected	Detected	Detected	Detected	Not Detected
(10^{-8})	0	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected
(10^{-9})	0	Not Detected	NOT DONE*	Not Detected	Not Detected	Not Detected

* Highest and lowest DNA dilution were omitted

** Possible processing error; not repeated as dilutions on either side detected MTB DNA

3.1.4 Method 1 – FastDNA DNA extraction followed by the Xpert Tube Fill protocol on decontaminated stool specimens

DNA was extracted manually from the remainder of the decontaminated stool suspension from 13 participants (resuspended sediment) leftover from Method A and molecular detection was done using the Xpert Tube Fill assay. The sensitivity and specificity of the Xpert assay were 28.6% and 100% compared to the composite reference standard, 40.0% and 100% compared to the first respiratory culture only and 50.0% and 100% compared to the first respiratory Xpert (Table 3.4). The PPV was 100.0% for all reference standards whilst the NPV was the lowest for the composite reference standard at 54.6% and increased to 72.7% and 81.8% for the first respiratory culture and Xpert respectively. No indeterminate results were generated using this method.

Table 3.4. Method 1 Results summary

Method 1: Decontaminated stool + DNA extraction + Xpert Tube Fill	**Composite Reference Standard**		**First Respiratory Culture**		**First Respiratory Xpert**	
Result	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE
POSITIVE	2	0	2	0	2	0
NEGATIVE	5	6	3	8	2	9
INDETERMINATE	0	0	0	0	0	0
TOTAL	**7**	**6**	**5**	**8**	**4**	**9**
Sensitivity	**28.6**		**40.0**		**50.0**	
Specificity	**100**		**100**		**100**	

3.1.5 Method 2 – FastDNA DNA extraction followed by the Xpert Tube Fill protocol on untreated raw stool specimens

DNA was extracted manually from 32 raw stool specimens and molecular detection was done using the Xpert Tube Fill assay. The sensitivity and specificity of the Xpert assay compared to the composite reference standard were 16.7% and 70.8%; and 0.0% and 70.4% compared to both the first respiratory culture and Xpert (n = 31) (Table 3.5). The PPV was the highest for the composite reference standard at 12.5% and decreased for the first respiratory culture and Xpert to 0.0%. The NPV was the lowest for the composite reference standard at 77.3% and increased to 90.5% the first respiratory culture and Xpert. The indeterminate rate was 2/32 (6.3%).

Table 3.5. Method 2 Results summary

Method 2: Untreated stool + DNA extraction + Xpert Tube Fill	**Composite Reference Standard**		**First Respiratory Culture**		**First Respiratory Xpert**	
Result	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE
POSITIVE	1	7	0	8	0	8
NEGATIVE	5	17	2	19	2	19
INDETERMINATE	0	2	0	2	0	2
TOTAL	**6**	**26**	**2**	**29**	**2**	**29**
Sensitivity	**16.7**		**0.0**		**0.0**	
Specificity	**70.8**		**70.4**		**70.4**	

3.1.6 Method 3 – QIAamp DNA extraction followed by the Xpert Tube Fill protocol on lyophilised stool specimens

DNA was manually extracted from 63 lyophilised stool specimens and molecular detection was done using the Xpert Tube Fill assay. The sensitivity and specificity compared to the composite reference standard were 37.0% and 88.9%; 44.4% and 86.7% compared to the first respiratory culture and 66.7% and 88.2% compared to the first respiratory Xpert (Table 3.6). The PPV was the highest for the composite reference standard at 71.4% and decreased for the first respiratory culture and Xpert to 57.1%. The NPV was the lowest for the composite reference standard at 65.3% and increased to 79.6% and 91.8% for the first respiratory culture and Xpert respectively. No indeterminate results were observed (0.0%).

Table 3.6. Method 3 Results summary

Method 3: Lyophilisation + Xpert Tube Fill	**Composite Reference Standard**		**First Respiratory Culture**		**First Respiratory Xpert**	
Result	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE
POSITIVE	10	4	8	6	8	6
NEGATIVE	17	32	10	39	4	45
INDETERMINATE	0	0	**0**	**0**	**0**	**0**
TOTAL	**27**	**36**	**18**	**45**	**12**	**51**
Sensitivity	**37.0**		**44.4**		**66.7**	
Specificity	**88.9**		**86.7**		**88.2**	

3.1.7 Method 4 – QIAamp DNA extraction followed by the FluoroType protocol on lyophilised stool specimens

Only 8 stool samples were tested using this method. DNA was manually extracted from the lyophilised stool and molecular detection was done using the HAIN FluoroType platform. Of the 8 specimens tested, 6 gave indeterminate results (75.0%). The remaining 2 specimens tested positive on the index method as well as all the reference standards. Sensitivity and specificity calculations were not done due to the small number of samples included.

3.1.8 Method 5 – GenoXtract DNA extraction followed by the FluoroType protocol on lyophilised stool specimens

DNA was extracted automatically from the lyophilised stool of 33 participants; and molecular detection was done using the HAIN FluoroType platform. The sensitivity and specificity compared to the composite reference standard were 75.0% and 85.7%; 75.0% and 63.6% compared to the first respiratory culture and 71.4% and 58.3% compared to the first respiratory Xpert (Table 3.7). The PPV was the highest for the composite reference standard at 90.0% and decreased for the first respiratory culture to 60.0% and to 50.0% for the first respiratory Xpert. The NPV was the lowest for the composite reference standard at 66.7% and increased to 77.8% for both the first respiratory culture and Xpert. The indeterminate rate was very high at 14/33 (42.4%).

Table 3.7. Method 5 Results summary

Method 5: Lyophilisation + GXT + FluoroType	**Composite Reference Standard**		**First Respiratory Culture**		**First Respiratory Xpert**	
Result	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE
POSITIVE	9	1	6	4	5	5
NEGATIVE	3	6	2	7	2	7
INDETERMINATE	7	7	6	8	3	11
TOTAL	**19**	**14**	**14**	**19**	**10**	**23**
Sensitivity	**75.0**		**75.0**		**71.4**	
Specificity	**85.7**		**63.6**		**58.3**	

3.1.9 Method 6 – GenoXtract DNA extraction followed by the Xpert Tube Fill protocol on lyophilised stool specimens

DNA was extracted using the automated GenoXtract DNA extraction protocol from the lyophilised stool of 49 participants and molecular detection was done using the Xpert Tube Fill platform. The sensitivity and specificity compared to the composite reference standard were 15.0% and 100%; 23.1% and 100% compared to first respiratory culture and 37.5% and 100.0% compared to the first respiratory Xpert (Table 3.8). The PPV was 100.0% for all reference standards whilst the NPV was the lowest for the composite reference standard at 63.0% and increased to 78.3% and 89.1% for the first respiratory culture and Xpert respectively. No indeterminate results were observed (0.0%).

Table 3.8. Method 6 Results summary

Method 6: Lyophilisation + GXT + Xpert Tube Fill	Composite Reference Standard		First Respiratory Culture		First Respiratory Xpert	
Result	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE
POSITIVE	3	0	3	0	3	0
NEGATIVE	17	29	10	36	5	41
INDETERMINATE	0	0	0	0	0	0
TOTAL	**20**	**29**	**13**	**36**	**8**	**41**
Sensitivity	**15.0**		**23.1**		**37.5**	
Specificity	**100.0**		**100.0**		**100.0**	

3.2 Indeterminate rates

The error rates for all methods are summarised in Figure 3.2. The FluoroType detection platform from Method 4 and Method 5 showed the highest indeterminate rates and the lowest indeterminate rates were seen in Methods 1, 3 and 6 respectively.

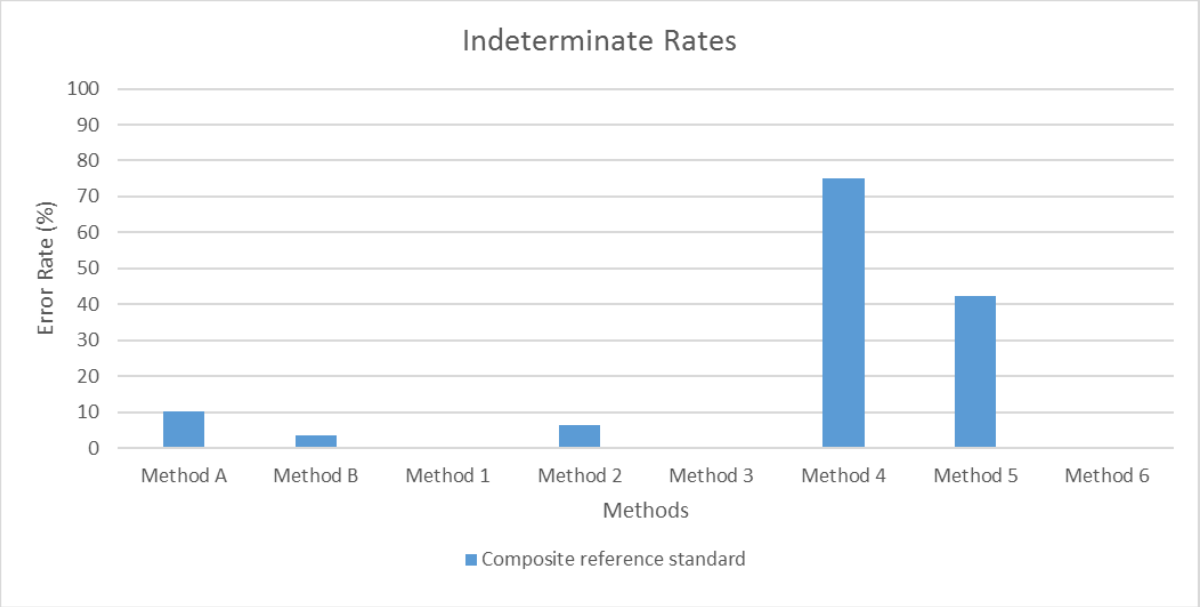

Figure 3.2. Indeterminate rates for all methods

3.3 Overview of results

In our setting, any child with suspected PTB would have at least one GA specimen, or ES in older children, tested by routine smear, culture and Xpert MTB/RIF (NHLS Tygerberg). We therefore compare the performance of the various index methods compared to the first respiratory culture (gold standard) result (Table 3.9).

Table 3.9. Summary of method sensitivities as compared to the secondary reference standard (First respiratory culture) and indeterminate rates

Method	Sample Size	Sensitivity	Specificity	Indeterminate Rate	Detection Platform
A	n = 166	47.1%	97.8%	10.3%	Xpert MTB/RIF
B	n = 196	45.8%	98.2%	3.4%	Xpert MTB/RIF
1	n = 13	40.0%	100%	0.0%	Xpert Tube Fill
2	n = 31	0.0%	70.4%	6.3%	Xpert Tube Fill
3	n = 63	44.4%	86.7%	0.0%	Xpert Tube Fill
4	n = 8	Not Calculated*	Not Calculated*	75.0%	FluoroType
5	n = 33	75.0%	63.6%	42.4%	FluoroType
6	n = 49	23.1%	100%	0.0%	Xpert Tube Fill

*Sensitivity and specificity not calculated due to small numbers of specimens tested

Chapter 4: Discussion

Stool is an attractive alternative specimen type for the laboratory diagnosis of TB in paediatric patients. Previous studies have shown that the TB bacilli can remain intact and survive the passage through the digestive system (88). The aim of the study was to evaluate the diagnostic utility of stool specimens using different protocols for the molecular detection of MTB in children investigated for suspected intrathoracic TB with or without extrathoracic TB.

The study was divided into two sections: firstly, to determine the diagnostic performance of the Xpert MTB/RIF assay on raw and decontaminated stool specimens; and secondly, to determine whether including DNA extraction steps from raw and/or lyophilised specimens would improve performance of the molecular assays.

Evaluation of new diagnostic methods for paediatric TB is complex as the reference standard (liquid culture) has poor sensitivity for paucibacillary TB (89). To improve on the reference standard, multiple respiratory specimens were collected by the study team and analysed by Xpert and culture. Although this strategy increases the number of cases confirmed bacteriologically, it also creates an unfair comparison for the index diagnostic strategy (molecular analysis of stool). Comparing the performance of Xpert and other molecular tests on a single stool specimen vs. the composite respiratory reference standard (up to 6 specimens) is biased and not a reflection of the current diagnostic practice.

In the initial phase of the study, stool specimens were collected from children for smear microscopy, liquid culture and Xpert MTB/RIF testing (Method A). Smear microscopy and liquid culture results were analysed in a larger ongoing cohort study (Walters E, unpublished data) and will not be discussed here. However, culture contamination rates were high. Stool contains many microorganisms forming part of the normal flora. A high stool culture contamination rate (greater than the generally accepted rate of 5-8% for liquid culture) compared to respiratory specimens was also observed in other stool studies (79,90,91). Smear microscopy and liquid culture were therefore discontinued and subsequent stool specimens were processed for direct Xpert MTB/RIF testing only, according to a study-specific protocol involving centrifugation and concentration steps (Method B). The protocol for the direct detection of MTB from stool (Method B) mainly focused on the removal of large particulate matter and possible PCR-inhibitors by centrifugation and buffered wash-steps while attempting to preserve a detectable amount of MTB DNA as well as its integrity. Method A used the stool supernatants for decontamination after the larger stool particles had been allowed to settle (gravitational settling) where method B used a brief centrifugation step (mechanical settling) to settle these particulates. The

supernatants from the gravitationally settled samples in Method A could have contained more PCR inhibitors and perhaps some unsettled particulates.

Individually, Method A detected MTB DNA in 11/195 (5.6%) participants, while Method B detected 14/208 (6.7%) from stool specimens tested on the Xpert MTB/RIF platform. The relatively wide range of starting stool mass for both protocols, 0.3 g-5 g depending on the amount of stool received from a participant, reduces the reproducibility of the protocols. In addition, both methods require centrifugation, making them impractical for implementation in most high-burdened settings and limiting the point-of-care application for the Xpert MTB/RIF assay.

The Xpert MTB/RIF assay on decontaminated and raw stool specimens (Method A and B) targeted intact bacilli (viable or non-viable) and showed a combined sensitivity of 45.8% - 47.1% (n = 403) compared to the first respiratory culture. Published data on the stool Xpert MTB/RIF assay report sensitivity between 47.1% - 100% compared to culture of up to 2 respiratory specimens (79–82). However, these studies only included small numbers of bacteriologically confirmed patients. When only considering the 2 larger studies (n = 267 and n = 115), the sensitivity range was narrower at 47.1% - 68.8% compared to respiratory specimen culture (80,81). This is similar to our finding, confirming the low sensitivity of Xpert MTB/RIF detection from stool specimens.

The indeterminate rate using intact bacilli as a target was 10.3% for Method A, but decreased to 3.4% when the decontamination step was omitted in Method B. The increased indeterminate rate in Method A may have been due to increased Xpert inhibition resulting from the concentration of the specimen into a sediment (following the decontaminating procedure) and resuspending into 3 ml phosphate buffer and aspirating 1 ml for Xpert, compared to Method B where 1 ml was aspirated from the middle layer of post-centrifuged stool/phosphate buffer supernatants (10 ml).

The range of specificity for both methods A and B against the CRS was 97.9 – 100%, while against the secondary reference standard the specificity was lower at 97.8% – 98.2%. Specificity below 100% may suggest false positive results for stool Xpert or may indicate that stool Xpert confirmed additional cases not detected on respiratory specimens. When considering the secondary reference standard, the specificity is lower because the reference standard itself missed cases detected on Xpert or culture of respiratory specimens other than the first one collected (2 for method A and 3 for method B). All 5 cases were positive on stool Xpert. Only one case was negative on all respiratory specimens (by both culture and Xpert) but positive on stool Xpert by method A. The child was diagnosed with TB based on clinic-epidemiological and radiological evidence and is therefore likely to have represented a true positive stool Xpert. In all cases, respiratory and stool specimens were collected before treatment

initiation. One participant for Method B had positive results on Xpert of both respiratory and stool specimens but was negative on all cultures. The child had had a previous episode of TB, but presented with new imaging features of neurotuberculosis and a worsening chest radiograph suggestive of active TB. Although it is debatable whether the chest radiology was indeed active TB, magnetic resonance imaging of the brain showed tuberculomas. The positive respiratory and stool Xpert, with negative respiratory cultures, may suggest that residual MTB DNA was present in respiratory secretions, and may not necessarily have indicated active pulmonary TB. However, the tuberculomas suggest active TB disease. In summary, our results suggest that stool Xpert may have an incremental diagnostic value over the reference standard (92).

Due to the high enzymatic content of intestinal secretions within stool specimens, it was hypothesised that the low sensitivity obtained with Method A and B may be due to the gastric environment degrading intact bacilli. Therefore, it was decided to extract DNA in an attempt to capture whole (intracellular) and extracellular MTB DNA fragments, thereby increasing the likelihood of reaching the limit of detection of the Xpert MTB/RIF assay (10).

DNA was extracted manually from direct raw stool specimens (Method 1 and 2), and tested on the Xpert Tube Fill assay. This novel Xpert protocol was used to bypass the internal washing and DNA extraction steps within the GeneXpert cartridge. The sensitivities for Method 1 and 2 compared to the first respiratory culture were 40.0% and 0.0% respectively. Even though the numbers are too small for definitive conclusions (n = 13 and n = 32), it is evident that the sensitivity of MTB detection did not improve using these methods. Method 2 did however detect 8 positive signals with a low specificity of 70.4%, suggesting the possibility of false positive results. From the suspected false positives, only 3/8 participants were started on antituberculosis treatment. While the indeterminate rate (6.3%) for Method 2 was similar to that seen in Method A and Method B, both indeterminate results were Xpert Tube Fill loading errors with insufficient remaining specimen to repeat the tests.

A major challenge of processing stool specimens for all methods was the variability in stool consistencies and following the same protocol regardless of water content i.e. liquid (diarrhoeic) or solid (constipated) stool specimens. Due to the difficulty in obtaining a reproducible starting mass for the DNA protocols, it was hypothesised that removing the water from the stool by lyophilisation would allow for easy-to-work-with specimen and in turn possibly concentrate any intact or extracellular MTB present in the specimen.

From published literature, lyophilisation has demonstrated an increased yield, and better quality of DNA for PCR-based studies using stool specimens obtained from pigs (93). Human studies looking at

other enteropathogens have also shown that lyophilisation significantly increases PCR-based detection of microorganisms from diarrheic stool specimens (94). From our own initial serial dilution experiments, we demonstrated that MTB DNA could be detected successfully from lyophilised stool specimens confirming that the DNA can remain intact following lyophilisation.

Overall, DNA extraction from lyophilised stool specimens (Methods 3-6) detected 14/63, 2/8, 10/33 and 3/49 instrument positive results on the respective detection platforms. Lyophilisation prior to manual DNA extraction and detection by Xpert Tube Fill (Method 3) showed a sensitivity of 44.4% compared to the first respiratory culture. The specificity of 86.7% suggested false positive results. However, 5/6 participants positive on stool but negative on culture/Xpert of respiratory specimens were started on antituberculosis treatment (after specimen collection). A second detection platform, the HAIN FluoroType assay, was considered as an alternative to the Xpert Tube Fill. There are no published data on the use of the automated FluoroType assay using stool specimens. Sputum data from adults, have reported a sensitivity of 100% for the FluoroType MTB for smear-positive participants, but only 56.3% for smear-negative participants from a low-incidence setting (84).

Detection of MTB DNA using the FluoroType assay from manually extracted DNA (Method 4) had an extremely high indeterminate rate of 75.0%. Although only a small number of specimens were tested by this method, due to the large costs involved per specimen and the high indeterminate rate, a different DNA extraction method was used. The HAIN GenoXtract DNA extraction protocol was tested in combination with the FluoroType assay.

The GenoXtract was used for DNA extraction from lyophilised stool specimens (Method 5). This method showed a sensitivity 75.0% when compared to the first respiratory culture. Although the low specificity of 63.6% suggested false positives, 3/4 of these participants were started on antituberculosis treatment. However, the indeterminate rate remained very high at 42.4%. Having concluded that the high indeterminate rate was due to the FluoroType detection assay, DNA extracted by GenoXtract from lyophilised stool specimens was tested on the Xpert Tube Fill detection platform (Method 6). The Xpert Tube Fill had a sensitivity of 23.1% compared to the first respiratory culture. The specificity however was the highest of all the methods tested at 100% (n = 49).

A limitation of this study was that no direct comparisons could be made between methods as the different methods were not assessed in parallel on the same participants. A further limitation is the choice of reference standard. There is no established protocol for MTB detection in stool specimens, which could serve as an adequate reference for the novel detection methods presented in this thesis. Respiratory specimen mycobacteriology was therefore chosen as the most objective microbiological

standard available. Although it is established that both Xpert and culture are imperfect diagnostic tests for paediatric TB, due to their relatively low sensitivity to confirm paucibacillary TB, comparing our stool methods to clinical reference standards would require complex clinical analyses which are beyond the scope of this work. However, using both a CRS which was inclusive of all mycobacteriology results from respiratory specimens, and a secondary reference which constituted a fairer comparison, enabled us to better explain which stool protocols had low specificity due to the reference being less sensitive than the index test. We were able to identify which protocols had an incremental value over the reference test, and which protocols resulted in possible false positive results.

In summary, we were not able to develop a diagnostic protocol that could detect MTB from paediatric stool samples with sufficient sensitivity. Direct Xpert MTB/RIF on stool without prior decontamination was the protocol with the best results for both sensitivity/specificity and invalid rate. In addition, direct stool Xpert detected additional cases not identified by the secondary reference standard. Some stool Xpert protocols published after this work was completed have demonstrated better sensitivity than our methods and warrant further study and validation in larger clinical cohorts (80,83). Although the Fluorotype platform demonstrated the best sensitivity, the high proportion of invalid results does not make this assay suitable for clinical application. Conversely, methods which demonstrated high specificity had sensitivity which was too low for clinical use.

Chapter 5: Conclusion

Stool specimens can easily be collected in routine settings as an alternative specimen type for the diagnosis and/or confirmation of paediatric TB. Lyophilisation can be used to concentrate any type of stool specimen without severely affecting DNA integrity. MTB DNA can be successfully isolated from stool specimens and can subsequently be detected by the HAIN FluoroType, Xpert MTB/RIF and Xpert Tube Fill molecular detection platforms. The availability of more sensitive molecular detection tools could possibly increase the utility of using stool specimens for rapid TB diagnosis in children. Given the current lack of an optimised processing method for the detection of TB from stool specimens, further research is required for stool to become a quality, reliable and minimally invasive specimen type for TB diagnosis. Stool processing methods are labour intensive and combined with the increased potential of containing PCR-inhibitors, careful processing strategies will need to be developed. Patients with previous TB will require special consideration before a confirmatory diagnosis can be made using only molecular techniques as specimens may contain residual MTB DNA and may result in false positive test results. The high rate of HIV and TB co-infection, and its impact on childhood mortality in high-burden settings, dictates the need for accurate, rapid and cost effective diagnostic strategies.

Appendices

Appendix A

Copy of ethics approval letter

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05 October 2011 **MAILED**

Dr E Walters
Department of Paediatrics
Tygerberg Hospital
Francie van Zijl Drive
Parow Valley
7500

Dear Dr Walters

Diagnostic Yield and Treatment Response in Childhood Intra-Thoracic Tuberculosis: Effect of Disease Severity.

ETHICS REFERENCE NO: N11/08/282

RE : APPROVAL

It is a pleasure to inform you that the Health Research Ethics Committee has approved the above-mentioned project at a meeting on 5 October 2011, including the ethical aspects involved, for a period of one year from this date.

This project is therefore now registered and you can proceed with the work. Please quote the above-mentioned project number in ALL future correspondence.

Please note a template of the progress report is obtainable on www.sun.ac.za/rds/ and should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly and subjected to an external audit.

Translations of the consent document in the languages applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Hélène Visser at City Health (Helene.Visser@capetown.gov.za Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

Approval Date: 5 October 2011 Expiry Date: 5 October 2012

Verbind tot Optimale Gesondheid · Committed to Optimal Health
Afdeling Navorsingsontwikkeling en -steun · Division of Research Development and Support
Posbus/PO Box 19063 · Tygerberg 7505 · Suid-Afrika/South Africa
Tel.: +27 21 938 9075 · Faks/Fax: +27 21 931 3952

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Ethics Letter

12-Feb-2016

Ethics Reference #: N11/09/282
Title: Diagnostic Yield and Treatment Response in Childhood Intra-Thoracic Tuberculosis: Effect of Disease Severity.

Dear Dr. Elisabetta Walters,

The HREC approved the following progress report by expedited review process:

Progress Report dated 01/12/2014 - 01/12/2015

The approval of this project is extended for a further year

Approval date: 11 February 2016

Expiry date: 10 February 2017

If you have any queries or need further help, please contact the REC Office 219389819.

Sincerely,

REC Coordinator
Ashleen Fortuin
Health Research Ethics Committee 2

Appendix B

Buffers and reagents

4% Sodium Bicarbonate solution (100 ml)

$NaHCO_3$ (Sodium bicarbonate) powder	4 g
Distilled water	100 ml

10x TE buffer (500 ml)

1 M Tris (hydroxymethyl) aminomethane pH 8.0	50 ml
0.5 M EDTA (Diaminoethane tetraacetic acid) pH 8.0	10 ml
Distilled water	440 ml

Autoclave (121 °C; 15 minutes)

BBL OADC Middlebrook enrichment media

Bovine albumin	50 g
Dextrose	20 g
Oleic Acid	0.6 g
Catalase	0.03 g

BBL PANTA

Polymyxin B	6000 units
Amphotericin B	600 µg
Nalidixic acid	2.4 mg
Trimethoprim	600 µg
Azlocillin	600 µg

NALC-NaOH-sodium citrate

Prepared fresh daily

NaOH 5%	50 ml
0.1 M trisodium citrate $3H_2O$	50 ml
NALC powder	0.5 g

Dissolved into 900 ml distilled water for a sodium hydroxide concentration of 2.5%

Phosphate buffered saline (PBS)

0.067M disodium phosphate (A):	
Sodium monohydrogen phosphate	9.47 g
Distilled water	1000 ml
0.067M monopotassium phosphate (B):	
Potassium dihydrophosphate	9.07 g
Distilled water	1000 ml

Add equal volumes of solution A and B and adjust pH to 6.8

Equipment used

Biological safety cabinet	LAB&AIR
Heating block	LASEC SA
Centrifuge (Heraeus Varifuge F)	Thermo Fischer Scientific
BACTEC MGIT 960 system	Becton Dickinson
Light and fluorescent microscope	OlympusX41
GenoXtract (HAIN)	NorDiag ASA
BP 211 D scale (d = 0.01 mg)	Sartorius
Water bath – (Memmert)	LASEC SA

Appendix C

FastDNA® Spin for Soil Kit (MP biomedicals) Protocol

1. Add 0.1 g sample (stool) to Lysing Matrix E Tube
2. Add 978 µl Sodium Phosphate Buffer and 122 µl MT buffer
3. Secure tubes in the Ribolyser Instrument and process for 2 x 40 seconds at speed 6.0
4. Centrifuge Lysing Matrix E tube at 18,407xg for 15 minutes to pellet debris
5. Transfer supernatant to clean 2 ml tube, add 250 µl PPS reagent and shake tube by hand 10 times
6. Centrifuge at 18,407xg for 5 minutes to pellet precipitate, transfer supernatant to clean 15 ml tube. Add 1 ml Binding Matrix suspension to the supernatant
7. Invert by hand for 2 minutes to allow binding of DNA to matrix, Place tubes in rack for 3 minutes to settle Silica Matrix
8. Remove 500 µl supernatant and discard, resuspend Binding Matrix in remaining supernatant and transfer 700 µl to a SPIN™ Filter and centrifuge at 18,407xg for 1 minute. Empty the catch tube and add remaining supernatant to the SPIN™ Filter and spin again.
9. Add 500 µl SEWS-M to the SPIN™ Filter and gently resuspend the pellet using pipette tip (ensure ethanol has been pre-added to concentrated SEWS-M
10. Centrifuge at 18,407xg for 1 minute; discard the flow through and any SEWS-M which did not pass through the matrix. Centrifuge for 2 minutes to remove residual SEWS-M wash solution
11. Remove SPIN™ Filter and place in a new Catch Tube. Air dry for 5 minutes at room temperature
12. Add 200 µl* DES (DNA/Pyrogen Free Water) to the binding matrix and gently stir with a finger flick to resuspend the silica. Incubate for 5 minutes at 55°C
13. Centrifuge at 18,407xg for 1 minute to transfer eluted DNA to the catch tube

* Protocol suggested 100 µl DES was increased to 200 µl to accommodate downstream protocols.

Appendix D

QIAamp® Fast DNA Stool Mini Kit protocol

Isolation of DNA from stool for pathogen detection

1. Weigh 180-220 mg* stool in a 2 ml microcentrifuge tube and place tube on ice
2. Add 1 ml InhibitEX buffer to each stool specimen, vortex continuously for 1 minute or until the stool specimen is thoroughly homogenised
3. Heat the suspension for 5 minutes at 95°C**, vortex for 15 seconds
4. Centrifuge specimen for 1 minute to pellet stool particles
5. Pipet 15 µl Proteinase K into a new 1.5 ml microcentrifuge tube
6. Pipet 200 µl supernatant from step 4 into the 1.5 ml microcentrifuge tube
7. Add 200 µl Buffer AL and vortex for 15 seconds. (Do NOT add Buffer AL directly to the Proteinase K)
8. Incubate at 70°C for 10 minutes
9. Add 200 µl of ethanol (96-100%) to the lysate, mix by vortexing
10. Carefully apply 600 µl lysate from step 9 to the QIAamp spin column. Close the cap and centrifuge for 1 minute. Place the QIAamp spin column in a new 2 ml collection tube and discard the collection tube containing the filtrate
11. Carefully open the QIAamp spin column and add 500 µl Buffer AW1. Centrifuge for 1 minute. Place QIAamp spin column in a new 2 ml collection tube and discard the collection tube containing the filtrate
12. Carefully open the QIAamp spin column and add 500 µl Buffer AW2. Centrifuge for 3 minute. Discard the collection tube and the filtrate
13. Place QIAamp spin column in a new 2 ml collection tube. Centrifuge for 3 minutes
14. Transfer the QIAamp spin column in a new labelled 1.5 ml microcentrifuge tube. Pipette 200 µl Buffer ATE directly onto the QIAamp membrane. Incubate for 1 minute at room temperature. Centrifuge for 1 minute to elute DNA

* For lyophilised stool specimens 100 mg was used as starting material

** The lysis temperature was increased from 70°C to 95°C for difficult to lyse cells (Gram-positive bacteria)

Appendix E

GenoXtract Stool Extraction Kit VER 2.0 (HAIN) Protocol

Kit for automated extraction of DNA from stool samples and rectal smears using the GenoXtract extraction instrument

Preparation of stool specimens

1. Using a swab, remove approximately 100 mg of stool specimen
2. Elute swab for 10 seconds with 1 ml Stool Stabilizer into a 2 ml screw cap tube
3. Vortex the specimen on a VV3 vortex at maximum speed for 5 minutes
4. Centrifuge at 5000xg for 4 minutes in a standard table top centrifuge
5. Transfer 500 µl of the supernatant to a 2 ml screw cap tube

Preparation and start of GenoXtract instrument

1. Assemble tips and pumps
2. Turn on instrument and press “CONTINUE” after initialisation press “START PROTOCOL”
3. Select protocol “GXT_Stool_DNA_V2” from the protocol list
4. Select 200 µl as elution volume
5. Mount required number of pump-tip units onto instrument; Press “OK”
6. Open the required amount of foil covered Reagent Cartridges by rolling the Piercing Tool backwards and forwards several times with sufficient pressure. Ensure that all the wells are clearly opened
7. Promptly load the opened Reagent Cartridges to the GenoXtract rack; Press “OK”
8. Place specimen tubes from step 5 of preparation of stool specimens in corresponding position in the GenoXtract rack; Press “OK”
9. Place required number of sterile 1.5 ml screw cap tubes for eluate in corresponding position in the GenoXtract rack; Press “OK”
10. Close the door; Press “START”

When the protocol is complete the touch-screen confirms that the run was successful. The eluate can be removed and is now ready for amplification.

Reagent Cartridge (GenoXtract Stool DNA)

Well 1: empty (located in the heating block in the GenoXtract)

Well 2: 2600 µl Elution buffer

Well 3: 2600 µl Water

Well 4: 2600 µl Water

Well 5: 900 µl Wash Buffer II (65-80% ethanol)

Well 6: 2500 µl Wash Buffer I (70-96% 2-propanol)

Well 7: empty

Well 8: 300 µl Magnetic Bead Solution

Well 9: Empty

Well 10: Empty

Well 11: 1600 µl Lysis Buffer (40-55% guanidine thiocyanate, 10-20% triton X-100)

Well 12: 500 µl (70-96% 2-proponol)

Stool Stabilizer contains 15-20% ammonium chloride, 1-5% cetrimonium bromide

Appendix F

FluoroType® MTB protocol

Molecular Genetic Assay for detection of the MTBC from patient specimens using the FluoroCycler® Instrument.

DNA Extraction

For automated DNA extraction from patient specimens, the GenoXtract instrument in combination with the GXT Stool Extraction kit can be used (Appendix E).

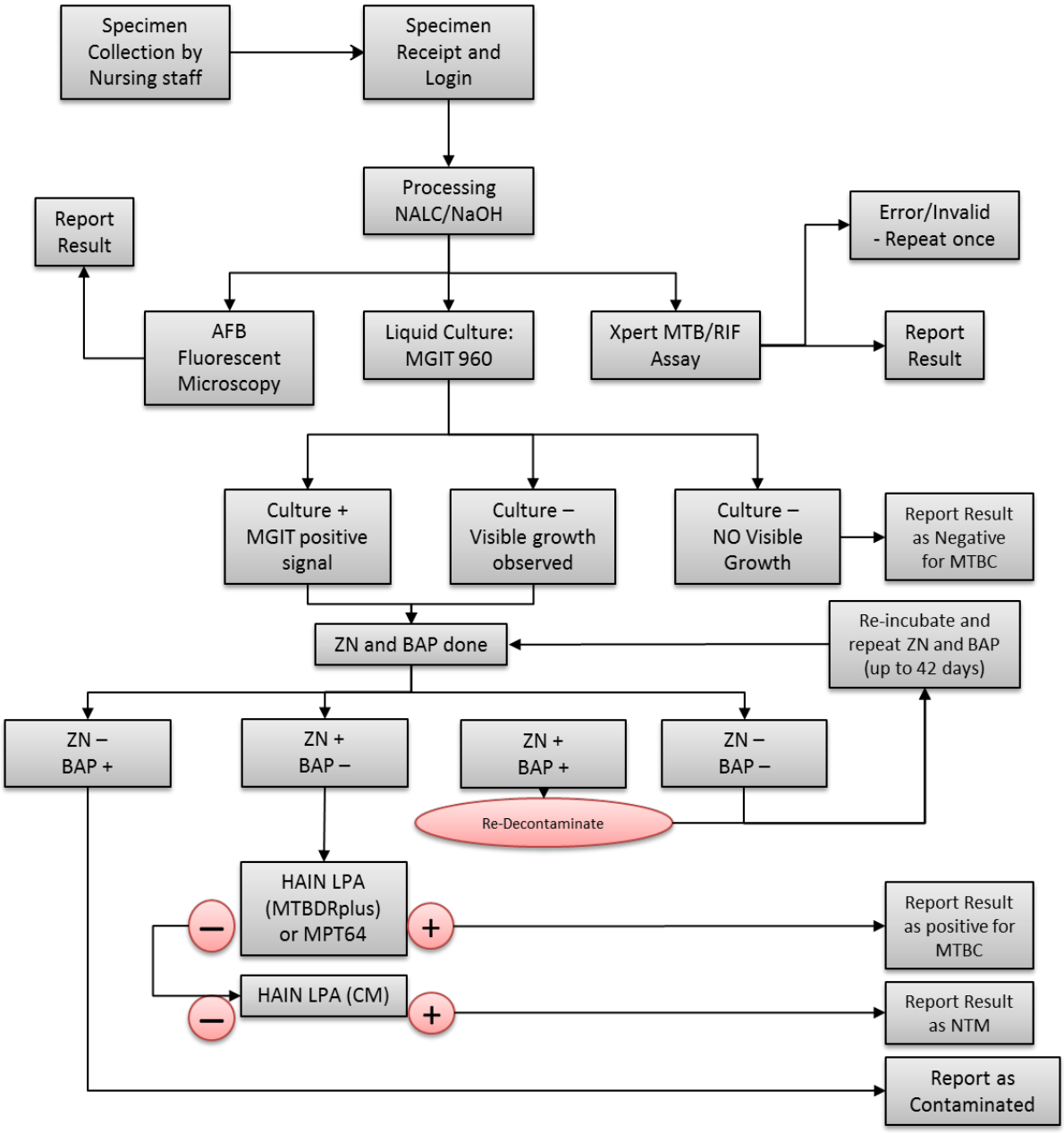
Amplification and detection

All reagents required for amplification such as polymerase and primers are included in the amplification Mixes A and B (AM-A and AM-B) and are optimized for this test.

1. After thawing, stir AM-A and AM-B carefully and spin down AM-A
2. Prepare for each specimen: 3 µl AM-A
   7 µl AM-B
   6 µl DNA solution
   Final Volume: 16 µl
3. Prepare a master mix containing AM-A and AM-B and mix carefully (Do NOT vortex)
4. Aliquot 10 µl of master mix into each PCR tube
5. Add 6 µl DNA solution in a separate area to each aliquot
6. Switch on the FluoroCycler®, start the Fluoro-Software (version 1.0.0 or higher) on the connected computer
7. Select the IVD mode and proceed as described in operator's manual
8. Starting at position 1 (leftmost position) place the prepared specimens in the FluoroCycler®; If less than 12 specimens, add an empty reaction tube in position 12 (rightmost position) to ensure proper closure of heating lid
9. Select the "FluoroMicrobial1 PCR" protocol from the "Protocol" page of the Fluoro-Software
10. Enter detailed specimen information on the "Samples" page
11. Choose "FT_MTB" as test
12. Start the run

Appendix G

General laboratory flow diagram (overview of NALC/NaOH processing)



Appendix H

GeneXpert MTB/RIF protocol

Procedure – Sputum Sediments

Note: Do not accept specimens with obvious food particles or other solid particulates.

Note: Process only as many samples at one time as there are modules available to run the test on the GeneXpert Dx System.

Guidelines for handling TB should be closely followed.

Phosphate/H2O buffer can be tested using Xpert MTB/RIF. Once the resuspension is prepared for standard laboratory smear or culture tests, ensure at least 0.5 ml of resuspended sediment is available to run Xpert MTB/RIF.

1. Label each Xpert MTB/RIF cartridge with the sample ID. (Write on the sides of the cartridge or affix ID label.)
2. Transfer at least 0.5 mL of the total resuspension pellet to a conical, screw-capped tube for the Xpert MTB/RIF using a sterile
3. Store re-suspended sediments at 2–8 °C if they are not immediately processed for Xpert MTB/RIF. Do not store for more than 12 hours
4. Add 1.5 ml of Xpert MTB/RIF Sample Reagent (SR) to 0.5 mL (Ratio 3:1) of resuspended sediment sample using a sterile transfer pipette and shake vigorously 10 – 20 times. Note: One back-and-forth movement is a single shake*
5. Incubate the specimen for 15 minutes at room temperature. At one point between 5 and 10 minutes of the incubation, again shake the specimen vigorously 10 – 20 times. Samples should be liquefied with no visible clumps of sputum. Particulate matter may exist that is not part of the sample

* In this study we used 2.0 ml Sample reagent and 1.0 ml of resuspended specimen ensuring that the ratio of 3:1 was maintained.

Appendix I

SANAS accreditation for NHLS TB laboratory

CERTIFICATE OF ACCREDITATION

In terms of section 22(2) (b) of the Accreditation for Conformity Assessment, Calibration and Good Laboratory Practice Act, 2006 (Act 19 of 2006), read with sections 23(1), (2) and (3) of the said Act, I hereby certify that:-

NATIONAL HEALTH LABORATORY SERVICE (NHLS)

Practice No.: PR 5200296

TYGERBERG ACADEMIC LABORATORY

Facility Accreditation Number: **M0390**

is a South African National Accreditation System accredited facility
provided that all conditions and requirements are complied with

This certificate is valid as per the scope as stated in the accompanying schedule of accreditation, Annexure "A", bearing the above accreditation number for

MEDICAL TESTING LABORATORY
CHEMISTRY, CYTOLOGY, ENDOCRINOLOGY, HAEMATOLOGY, HISTOLOGY, MICROBIOLOGY, SEROLOGY AND TUBERCULOSIS

The facility is accredited in accordance with the recognised International Standard

ISO 15189:2012

The accreditation demonstrates technical competency for a defined scope and the operation of a quality management system

While this certificate remains valid, the accredited facility named above is authorised to use the relevant accreditation symbol to issue facility reports and/or certificates

Mr R Josias
Chief Executive Officer

Effective Date: 11 December 2013
Certificate Expires: 10 December 2017

Facility Number: M0390

	Optochin	
	Oxidase	
	PYR	
	Satellitism	
	Spot Indole Test	
	XV Factors	
	Serological Identification Techniques:	Manual Method
	E.Coli O157 Serotyping	
	Pneumococcus Latex Test	
	Salmonella Sero-typing	
	Shigella Serotyping	
	Staphylococcus Agglutination	
	Streptococcus Agglutination	Manual Method
	Fungal Identification Techniques:	Manual Method
	Yeast	
	Germ Tube Test	
	Auxocolour	
	Moulds	
	Microscopy:	
	Lactophenol Cotton Blue Stain	
	Antimicrobial Susceptibility Testing:	Vitek II / Kirby Bauer
	Sensitivity Testing	
Organisms and Specimens	16S	Manual Method
Yeast, Moulds and all Specimens	Pan Fungal	Manual Method

Tuberculosis

Pulmonary and Extra Pulmonary Specimens	Direct Examination:	Manual Method
	Auramine Stain	
	Ziehl Neelsen	
	TB Culture	MGIT System
Sputum	Rapid TB Identification	Manual Method
Sputum	HAIN PCR for TB	Twincubator / Thermocycler
Respiratory Specimens	MTB / RIF Assay	GeneXpert

Appendix J

Example of specimen transport form including temperatures

DTTC Treatment Response Study

SAM 2 Sample Transport Log

Sample	Temp.	Destination
Resp.	2-8°C	NHLS TB Lab 9th Floor
NPA	2-8°C	Virology Lab 8th Floor Clinical Building
Stool	2-8°C	DTTC Mini Lab K0073
Urine	2-8°C	DTTC Mini Lab K0073
Blood	18-25°C	DTTC Mini Lab K0073
OMNI gene	18-25°C	NHLS TB Lab 9th Floor

1 FORM per TRANSPORT BOX. 1 BOX for samples with same transport temperature and same destination.

No	CHILD STUDY BARCODE	DTTC SAMPLE BARCODE (1 barcode per line)
1		SAMPLE BACRODE
2		SAMPLE BACRODE
3		SAMPLE BACRODE
4		SAMPLE BACRODE
5		SAMPLE BACRODE
6		SAMPLE BACRODE

DEPARTURE SITE (indicate with X):	ARRIVAL SITE (indicate with X):
1_Tygerberg Hospital G Ground	1_NHLS Tygerberg TB Lab (9th Floor)
2_Participant Home	2_NHLS Virology (8th Floor- Clinical Building)
3_Other, specify:	3_DTTC Mini Lab Room K0073
	-5_Other, specify:
	Received by:_____

Date departing	D	D	M	M	Y	Y	Date arriving	D	D	M	M	Y	Y
Time departing	H	H	H	M	M		Time arriving	H	H	H	M	M	
Temperature departing				°C			Temperature arriving				°C		

	RA CODE	DATE	SIGNATURE
TRANSPORTED BY		D D M M Y Y Y Y	
QC BY LAB TECH.		D D M M Y Y Y Y	
DATA CAPTURE 1		D D M M Y Y Y Y	
DATA CAPTURE 2		D D M M Y Y Y Y	

Treatment Response Study - Sample Transport Log – Version 1.2
17 August 2016 Page 1 of 1

Appendix K

Correspondence with Cepheid regarding sample volume added to the Xpert MTB/RIF assay

Bosch, C, Mnr <corneb@sun.ac.za>

From: Cepheid EU Tech Support <support@cepheideurope.com>
Sent: Wednesday, 06 September 2017 4:15 PM
To: Bosch, C, Mnr <corneb@sun.ac.za>
Cc: randall.farmer@cepheid.com
Subject: 000103810_Stellenbosch university_ Xpert sample/reagent volume [ref:_ 00D301549._50038tHCpu:ref]

Good day Corne,

Thank you for contacting Cepheid technical support. Apologies for the delay.

Regarding your inquiry, kindly note that the use of 3ml instead of the recommended 2ml with the Xpert MTB RIF assay will not have an effect on the result.
The exact amount of volume used is defined by the Assay Definition File, thus we recommend the use of at least 2ml.
Unfortunately the exact amount used is Proprietary Information and we are unable to provide any reference with this regard.

Thank you!
kind regards,

Kemoneilwe Modise
Technical Support Specialist
South Africa
Cepheid

FOR SERVICE & SUPPORT: www.cepheid.com/support TECH SUPPORT HOTLINE: +33 563 825 319 (South Africa : +27 861 22 76 35) CUSTOMER SERVICE HOTLINE +27 861 26 79 00 SOUTH AFRICA SWITCHBOARD +27 11 234 9636 FAX: +33 563 825 301 (South Africa : +27 11 234 9640) EMAIL Kemoneilwe.Modise@cepheid.com (Complaints and Inquiries: support@cepheideurope.com) WWW.CEPHEIDINTERNATIONAL.COM

mailto:Kemoneilwe.Modise@cepheid.co

Appendix L

Infection control for respiratory specimen collection

4.7.3 Collection of Respiratory Specimens (expectorated sputum, induced sputum and gastric aspirates)

- Outside (open air) space or a sputum booth located outside are the preferred spaces for obtaining expectorated sputum specimens and should be used wherever possible.
- For procedure rooms/cough rooms, the door should be locked or a sign put on the outside indicating that specimen collection is taking place inside, to avoid anyone entering the room during the procedure.
- All staff responsible for collecting respiratory specimens in the procedure rooms/cough rooms and performing gastric aspiration or lavage must wear personal protective equipment during throughout the procedure.
  - The personnel member collecting the specimen must wear a N95 respirator and disposable gloves.
  - In addition, a plastic apron should be worn for the procedure if contact with mucous or respiratory secretions is anticipated.
  - Caregivers assisting the staff with procedures (e.g. holding a child during specimen collection) should also be given particulate respirators to wear.
  - All personal protective equipment (masks, gloves, and aprons) should be discarded appropriately after completion of the procedure.
- Hand hygiene should be performed before and after participant contact (see SOP CM003 on hand washing).
- If the room provides at least 12 air changes per hour (ACH), the room must not be used for at least 35 minutes after the infectious patient has left the room, to allow enough air changes before another patient enters the room (see Table below from reference 7).
  - During this period, staff remaining in the room should continue to wear a N95 mask.
  - A fake clock with moveable hands should be fitted on the outside of the door, to mark the time when the room can next be used after specimen collection has been carried out. Allow at least 10 minutes after specimen collection, before exiting the room. Close the door immediately. Indicate the time when the next procedure can be done, on the fake clock on the door.
- After collection, specimen containers should be closed tightly and the outside of the container should be disinfected before it is labelled. The sample is then placed in a sample bag. All specimen bags should be placed in appropriate transport boxes at the appropriate temperature for transport to the laboratory.
- All surfaces should be cleaned before and after each procedure with appropriate disinfectant (see SOP CM006).

References

1. World Health Organization. Global Tuberculosis Report 2016. WHO. 2016;(Global TB Report 2016).

2. Raviglione MC, Uplekar MW. WHO's new Stop TB Strategy. Lancet. 2006;367(9514):952–5.

3. Zignol M, Sismanidis C, Falzon D, Glaziou P, Dara M, Floyd K. Multidrug-resistant tuberculosis in children: Evidence from global surveillance. Eur Respir J. 2013;42(3):701–7.

4. Falagas ME, Karageorgopoulos DE. Pandrug Resistance (PDR), Extensive Drug Resistance (XDR), and Multidrug Resistance (MDR) among Gram-Negative Bacilli: Need for International Harmonization in Terminology. Clin Infect Dis. 2008;46(7):1121–2.

5. Velayati AA, Masjedi MR, Farnia P, Tabarsi P, Ghanavi J, ZiaZarifi AH, et al. Emergence of new forms of totally drug-resistant tuberculosis bacilli: Super extensively drug-resistant tuberculosis or totally drug-resistant strains in Iran. Chest. 2009;136(2):420–5.

6. Sreeramareddy CT, Harsha Kumar HN, Arokiasamy JT. Prevalence of self-reported tuberculosis, knowledge about tuberculosis transmission and its determinants among adults in India: results from a nation-wide cross-sectional household survey. BMC Infect Dis. 2013;13:16.

7. von Delft A, Dramowski A, Khosa C, Kotze K, Lederer P, Mosidi T, et al. Why healthcare workers are sick of TB. Int J Infect Dis. 2015;32:147–51.

8. Churchyard GJ, Mametja LD, Mvusi L, Ndjeka N, Hesseling AC, Reid A, et al. Tuberculosis control in South Africa: Successes, challenges and recommendations. South African Med J. 2014;104(3):244–8.

9. Knechel NA. Tuberculosis: Pathophysiology, clinical features, and diagnosis. Crit Care Nurse. 2009;29(2):34–43.

10. Edwards D, Kirkpatrick CH. The immunology of mycobacterial diseases. Am Rev Respir Dis. 1986;134(5):1062–71.

11. Dunlap NE, Bass J, Fujiwara P, Hopewell P, Horsburgh CR, Salfinger M, et al. Diagnostic standards and classification of tuberculosis in adults and children. Vol. 161, American Journal of Respiratory and Critical Care Medicine. American Thoracic SocietyNew York, NY; 2000. p. 1376–95.

12. Dannenberg AM. Immune Mechanisms in the Pathogenesis of Pulmonary Tuberculosis. Rev Infect Dis. 1989;11:S369–78.

13. Lee JY. Diagnosis and treatment of extrapulmonary tuberculosis. Tuberc Respir Dis (Seoul). 2015;78(2):47–55.

14. Dierberg KL, Chaisson RE. Human immunodeficiency virus-associated tuberculosis: update on prevention and treatment. Clin Chest Med. 2013;34(2):217–28.

15. Lawn SD, Zumla AI. Tuberculosis. In: The Lancet. 2011. p. 57–72.

16. Chamie G, Luetkemeyer A, Walusimbi-Nanteza M, Okwera A, Whalen CC, Mugerwa RD, et al. Significant variation in presentation of pulmonary tuberculosis across a high resolution of CD4 strata. Int J Tuberc Lung Dis. 2010;14(10):1295–302.

17. Perez-Velez CM, Marais BJ. Tuberculosis in Children. N Engl J Med. 2012;367:348–61.

18. Marais BJ, Gie RP, Hesseling AC, Schaaf HS, Lombard C, Enarson DA, et al. A refined symptom-based approach to diagnose pulmonary tuberculosis in children. Pediatrics. 2006;118(5):e1350-9.

19. Oliwa JN, Karumbi JM, Marais BJ, Madhi SA, Graham SM, Walker C, et al. Tuberculosis as a cause or comorbidity of childhood pneumonia in tuberculosis-endemic areas: a systematic review. Lancet Respir Med. 2015;3(3):235–43.

20. Marais BJ, Pai M. New approaches and emerging technologies in the diagnosis of childhood tuberculosis. Paediatr Respir Rev. 2007;8(2):124–33.

21. Marais BJ, Gie RP, Hesseling AH, Beyers N. Adult-type pulmonary tuberculosis in children 10-14 years of age. Pediatr Infect Dis J. 2005;24(8):743–4.

22. Hesseling AC, Schaaf HS, Gie RP, Starke JR, Beyers N. A critical review of diagnostic approaches used in the diagnosis of childhood tuberculosis. Int J Tuberc Lung Dis. 2002;6(12):1038–45.

23. Swingler GH, Du Toit G, Andronikou S, Van Der Merwe L, Zar HJ, Swingler G. Diagnostic accuracy of chest radiography in detecting mediastinal lymphadenopathy in suspected pulmonary tuberculosis. Arch Dis Child. 2005;90:1153–6.

24. Theart AC, Marais BJ, Gie RP, Hesseling AC, Beyers N. Criteria used for the diagnosis of childhood tuberculosis at primary health care level in a high-burden, urban setting. Int J Tuberc Lung Dis. 2005;9(11):1210–4.

25. Burrill J, Williams CJ, Bain G, Conder G, Hine AL, Misra RR. Tuberculosis: A Radiologic Review. RadioGraphics. 2007 Sep;27(5):1255–73.

26. Pai M, Kalantri S, Dheda K. New tools and emerging technologies for the diagnosis of tuberculosis: part II. Active tuberculosis and drug resistance. Expert Rev Mol Diagn. 2006 May;6(3):423–32.

27. Drobac PC, Shin SS, Huamani P, Atwood S, Furin J, Franke MF, et al. Risk Factors for In-Hospital Mortality Among Children With Tuberculosis: The 25-Year Experience in Peru. Pediatrics. 2012;130(2).

28. Mandalakas AM, Detjen AK, Hesseling AC, Benedetti A, Menzies D. Interferon-gamma release assays and childhood tuberculosis: systematic review and meta-analysis [Review article]. Int J Tuberc Lung Dis. 2011;15(8):1018–32.

29. Lobato MN, Loeffler AM, Furst K, Cole B, Hopewell PC, Lobato M, et al. Detection of Mycobacterium tuberculosis in gastric aspirates collected from children: hospitalization is not necessary. Pediatrics. 1998;102(4):E40.

30. Marais BJ, Pai M. Specimen collection methods in the diagnosis of childhood tuberculosis. Indian J Med Microbiol. 2006;24(4):249–51.

31. Stockdale AJ, Duke T, Graham S, Kelly J. Evidence behind the WHO guidelines: hospital care for children: what is the diagnostic accuracy of gastric aspiration for the diagnosis of tuberculosis in children? J Trop Pediatr. 2010;56(5):291–8.

32. Hatherill M, Hawkridge T, Zar HJ, Whitelaw A, Tameris M, Workman L, et al. Induced sputum or gastric lavage for community-based diagnosis of childhood pulmonary tuberculosis? Arch Dis Child. 2009;94(3):195–201.

33. Zar HJ, Hanslo D, Apolles P, Swingler G, Hussey G, Shingadia D, et al. Induced sputum versus gastric lavage for microbiological confirmation of pulmonary tuberculosis in infants and young children: a prospective study. Lancet. 2005;365(9454):130–4.

34. Zar HJ, Tannenbaum E, Apolles P, Roux P, Hanslo D, Hussey G, et al. Sputum induction for the diagnosis of pulmonary tuberculosis in infants and young children in an urban setting in South Africa. 2000;305–8.

35. Zar HJ, Workman L, Isaacs W, Munro J, Black F, Eley B, et al. Rapid Molecular Diagnosis of Pulmonary Tuberculosis in Children Using Nasopharyngeal Specimens. 2012;1–8.

36. Marais BJ, Hesseling AC, Gie RP, Schaaf HS, Enarson DA, Beyers N. The Bacteriologic Yield in Children with Intrathoracic Tuberculosis. Clin Infect Dis. 2006;42(8):e69–71.

37. Zar HJ, Udwadia ZF. Advances in tuberculosis 2011–2012. Thorax. 2013;68:283–7.

38. Marais BJ, Brittle W, Painczyk K, Hesseling AC, Beyers N, Wasserman E, et al. Use of light-emitting diode fluorescence microscopy to detect acid-fast bacilli in sputum. Clin Infect Dis. 2008;47(2):203–7.

39. Steingart KR, Ramsay A. Optimizing sputum smear microscopy for the diagnosis of. 2007;327–31.

40. Palomino JC, Martin A, Portaels F, Cathode RM, Rüsch-Gerdes S, Pfyffer GE, et al. Evaluation of Automated BACTEC MGIT 960 System for Testing Susceptibility of. J Clin Microbiol. 2007;37(3):607–10.

41. Siddiqi SH, Rüsch-Gerdes S. For BACTEC™ MGIT 960™ TB System (Also applicable for Manual MGIT). 2006;

42. Rodrigues C, Shenai S, Sadani M, Sukhadia N, Jani M, Ajbani K, et al. Evaluation of the BACTEC MGIT 960 TB system for recovery and identification of Mycobacterium tuberculosis complex in a high through put tertiary care centre. Indian J Med Microbiol. 2009;27(3):217–21.

43. GLI. Mycobacteriology Laboratory Manual Editor ,1st ed. 2014;(April):1–154.

44. Muňoz-Sellart M, Yassin MA, Tumato M, Merid Y, Cuevas LE. Treatment outcome in children with tuberculosis in southern Ethiopia. Scand J Infect Dis. 2009;41(6–7):450–5.

45. Schaaf HS, Shean K DP. Diagnostic Delay , Clinical Features , and Outcome. 2003;1106–12.

46. Cepheid®. Xpert® MTB/RIF Package Insert Ref GXMTB/RIF-US-10. 2015;(Feb):1–48. Available from: http://www.cepheid.com/manageddownloads/xpert-mtb-rif-english-package-insert-301-1404-rev-b-february-2015.pdf

47. World Health Organization. Xpert MTB / RIF implementation manual. WHO Man. 2014;1–52.

48. Detjen AK, DiNardo AR, Leyden J, Steingart KR, Menzies D, Schiller I, et al. Xpert MTB/RIF assay for the diagnosis of pulmonary tuberculosis in children: A systematic review and meta-analysis. Lancet Respir Med. 2015;3(6):451–61.

49. Rufai SB, Kumar P, Singh A, Prajapati S, Balooni V, Singh S. Comparison of Xpert MTB / RIF with

Line Probe Assay for Detection of Rifampin-Monoresistant Mycobacterium tuberculosis. 2014;52(6):1846–52.

50. Brossier F, Veziris N, Truffot-pernot C, Jarlier V, Sougakoff W. Performance of the Genotype MTBDR Line Probe Assay for Detection of Resistance to Rifampin and Isoniazid in Strains of Mycobacterium tuberculosis with Low- and High-Level Resistance. 2006;44(10):3659–64.

51. Zhang Y, Yew W. Mechanisms of drug resistance in *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis. 2009;13(11):1320–30.

52. Barnard M, Warren R, Van Pittius NG, Van Helden P, Bosman M, Streicher E, et al. GenoType MTBDRs/ line probe assay shortens time to diagnosis of extensively drug-resistant tuberculosis in a high-throughput diagnostic laboratory. Am J Respir Crit Care Med. 2012;186(12):1298–305.

53. Theron G, Peter J, Richardson M, Barnard M, Donegan S, Warren R, et al. The diagnostic accuracy of the GenoType ® MTBDR sl assay for the detection of resistance to second-line anti-tuberculosis drugs (Review). 2014;(10).

54. Connell TG, Zar HJ, Nicol MP. Advances in the Diagnosis of Pulmonary Tuberculosis in HIV-Infected and HIV-Uninfected Children. J Infect Dis. 2011;204(suppl 4):S1151–8.

55. Gomez-pastrana D. Diagnosis of Pulmonary Tuberculosis in Children. J Infect Dis Ther. 2013;1:17–24.

56. Sharma SK, Kohli M, Yadav RN, Chaubey J, Bhasin D, Sreenivas V, et al. Evaluating the diagnostic accuracy of xpert MTB/RIF assay in pulmonary tuberculosis. PLoS One. 2015;10(10):1–9.

57. Walters E, Goussard P, Bosch C, Hesseling AC, Gie RP. GeneXpert MTB/RIF on bronchoalveolar lavage samples in children with suspected complicated intrathoracic tuberculosis: A pilot study. Pediatr Pulmonol. 2014;49(11):1133–7.

58. Oberhelman R a, Soto-Castellares G, Caviedes L, Castillo ME, Kissinger P, Moore D a J, et al. Improved recovery of Mycobacterium tuberculosis from children using the microscopic observation drug susceptibility method. Pediatrics. 2006;118(1):e100-6.

59. Oberhelman RA, Soto-Castellares G, Gilman RH, Caviedes L, Castillo ME, Kolevic L, et al. Diagnostic approaches for paediatric tuberculosis by use of different specimen types, culture methods, and PCR: A prospective case-control study. Lancet Infect Dis. 2010;10(9):612–20.

60. Nansumba M, Kumbakumba E, Orikiriza P, Muller Y, Nackers F, Debeaudrap P, et al. Detection Yield and Tolerability of String Test for Diagnosis of Childhood Intrathoracic Tuberculosis. Pediatr Infect Dis J. 2016;35(2):146–51.

61. Atwine D, Nansumba M, Orikiriza P, Riera M, Nackers F, Kamara N, et al. Intra-gastric string test: An effective tool for diagnosing tuberculosis in adults unable to produce sputum. Int J Tuberc Lung Dis. 2015;19(5):558–64.

62. Ligthelm LJ, Nicol MP, Hoek KGP, Jacobson R, van Helden PD, Marais BJ, et al. Xpert MTB/RIF for rapid diagnosis of tuberculous lymphadenitis from fine-needle-aspiration biopsy specimens. J Clin Microbiol. 2011;49(11):3967–70.

63. Grewal R, Abayomi AE. Bone marrow morphological features and diagnostic value in paediatric disseminated tuberculosis in the setting of increased HIV prevalence. South African Med J. 2013;103(5):326.

64. Minion J, Leung E, Talbot E, Dheda K, Pai M, Menzies D. Diagnosing tuberculosis with urine lipoarabinomannan: systematic review and meta-analysis. Eur Respir J. 2011;38(6):1398–405.

65. Lawn SD, Gupta-Wright A. Detection of lipoarabinomannan (LAM) in urine is indicative of disseminated TB with renal involvement in patients living with HIV and advanced immunodeficiency: evidence and implications. Trans R Soc Trop Med Hyg. 2016;110(3):180–5.

66. Kroidl I, Clowes P, Reither K, Mtafya B, Rojas-Ponce G, Ntinginya EN, et al. Performance of urine lipoarabinomannan assays for paediatric tuberculosis in Tanzania. Eur Respir J. 2015;46:761–70.

67. World Health Organization. The use of lateral flow urine lipoarabinomannan assay (LF-LAM) for the diagnosis and screening of active tuberculosis in people living with HIV. WHO. 2015;

68. Colebunders R, Nembunzu M, Portaels F, Lusakumunu K, Kapita B, Piot P. Isolation of mycobacteria from stools and intestinal biopsies from HIV seropositive and HIV seronegative patients with and without diarrhea in Kinshasa, Zaïre. Preliminary results. Ann la Société belge médecine Trop. 1990 Dec;70(4):303–9.

69. Donald PR, Schaaf HS, Gie RP, Beyers N, Sirgel FA, Venter A. Stool microscopy and culture to assist the diagnosis of pulmonary tuberculosis in childhood. J Trop Pediatr. 1996;42(5):311–2.

70. Kent P. Public health mycobacteriology : a guide for the level III laboratory. Atlanta Ga.: U.S. Dept. of Health and Human Services Public Health Service Centers for Disease Control; 1985.

71. Kokuto H, Sasaki Y, Yoshimatsu S, Mizuno K, Yi L, Mitarai S. Detection of Mycobacterium tuberculosis (MTB) in Fecal Specimens From Adults Diagnosed With Pulmonary Tuberculosis Using the Xpert MTB/Rifampicin Test. Open Forum Infect Dis. 2015;2(2):ofv074-ofv074.

72. El Khéchine A, Henry M, Raoult D, Drancourt M. Detection of Mycobacterium tuberculosis complex organisms in the stools of patients with pulmonary tuberculosis. Microbiology. 2009;155(7):2384–9.

73. Allen BW. Comparison of three methods for decontamination of faeces for isolation of Mycobacterium tuberculosis. Tubercle. 1991;72(3):214–7.

74. El Khéchine A, Drancourt M. Diagnosis of pulmonary tuberculosis in a microbiological laboratory. Med Mal Infect. 2011;41(10):509–17.

75. Portaels F, De Muynck A, Sylla MP. Selective isolation of mycobacteria from soil: a statistical analysis approach. J Gen Microbiol. 1988;134(3):849–55.

76. Cadmus SIB, Jenkins AO, Godfroid J, Osinusi K, Adewole IF, Murphy RL, et al. Mycobacterium tuberculosis and Mycobacterium africanum in stools from children attending an immunization clinic in Ibadan, Nigeria. Int J Infect Dis. 2009;13(6):740–4.

77. Cordova J, Shiloh R, Gilman RH, Sheen P, Martin L, Arenas F, et al. Evaluation of molecular tools for detection and drug susceptibility testing of Mycobacterium tuberculosis in stool specimens from patients with pulmonary tuberculosis. J Clin Microbiol. 2010;48(5):1820–6.

78. Wolf H, Mendez M, Gilman RH, Sheen P, Soto G, Velarde AK, et al. Diagnosis of pediatric pulmonary tuberculosis by stool PCR. Am J Trop Med Hyg. 2008;79(6):893–8.

79. Hillemann D, Rüsch-Gerdes S, Boehme C, Richter E. Rapid molecular detection of extrapulmonary tuberculosis by the automated genexpert MTB/RIF system. J Clin Microbiol. 2011;49(4):1202–5.

80. Marcy O, Ung V, Goyet S, Borand L, Msellati P, Tejiokem M, et al. Performance of Xpert MTB/RIF and Alternative Specimen Collection Methods for the Diagnosis of Tuberculosis in HIV-Infected Children. Clin Infect Dis. 2016;62(9):1161–8.

81. Nicol MP, Spiers K, Workman L, Isaacs W, Munro J, Black F, et al. Xpert MTB/RIF Testing of Stool Samples for the Diagnosis of Pulmonary Tuberculosis in Children. Clin Infect Dis. 2013;57(3):e18–21.

82. Walters E, Gie RP, Hesseling AC, Friedrich SO, Diacon AH, Gie RP. Rapid Diagnosis of Pediatric Intrathoracic Tuberculosis From Stool Samples Using the Xpert MTB/RIF Assay. Pediatr Infect Dis J. 2012;31(12):1316.

83. Banada PP, Naidoo U, Deshpande S, Karim F, Flynn JL, O'Malley M, et al. A Novel Sample Processing Method for Rapid Detection of Tuberculosis in the Stool of Pediatric Patients Using the Xpert MTB/RIF Assay. Rengarajan J, editor. PLoS One. 2016 Mar;11(3):e0151980.

84. Hofmann-Thiel S, Hoffmann H. Evaluation of Fluorotype MTB for detection of Mycobacterium tuberculosis complex DNA in clinical specimens from a low-incidence country. BMC Infect Dis. 2014;14(1):59.

85. World Health Organization. Tuberculosis Laboratory BioSafety Manual. WHO Man. 2012;

86. Genotype® MTBDRplus product insert. Version 2. Hain Lifescience [Internet]. 2012. Available from: https://www.ghdonline.org/uploads/MTBDRplusV2_0212_304A-02-02.pdf

87. Graham SM, Cuevas LE, Jean-philippe P, Browning R, Casenghi M, Detjen AK, et al. Clinical Case Definitions for Classification of Intrathoracic Tuberculosis in Children : An Update. 2015;61(Suppl 3).

88. Kotgire Santosh A. Microbiological stool examination: Overview. J Clin Diagnostic Res. 2012;6(3):503–9.

89. Zar HJ, Connell TG, Nicol M. Diagnosis of pulmonary tuberculosis in children: new advances. Expert Rev Anti Infect Ther. 2010;8(3):277–88.

90. Oramasionwu GE, Heilig CM, Udomsantisuk N, Kimerling ME, Eng B, Nguyen HD, et al. The utility of stool cultures for diagnosing tuberculosis in people living with the human immunodeficiency virus. Int J Tuberc Lung Dis. 2013;17(8):1023–8.

91. Hillemann D, Richter E, Rüsch-Gerdes S. Use of the BACTEC Mycobacteria Growth Indicator Tube 960 automated system for recovery of Mycobacteria from 9,558 extrapulmonary specimens, including urine samples. J Clin Microbiol. 2006;44(11):4014–7.

92. Theron G, Venter R, Calligaro G, Smith L, Limberis J, Meldau R, et al. Xpert MTB/RIF Results in Patients With Previous Tuberculosis: Can We Distinguish True From False Positive Results? Clin Infect Dis. 2016;62(8):995–1001.

93. Ruiz R, Rubio LA. Lyophilisation improves the extraction of PCR-quality community DNA from

pig faecal samples. J Sci Food Agric. 2009;89(4):723–7.

94. Donatin E, Drancourt M. Optimized microbial DNA extraction from diarrheic stools. BMC Res Notes. 2012;5(1):702.